BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF *Streptococcus pneumoniae* STRAINS RESISTANT TO BETA-LACTAM ANTIBIOTICS

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BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF *Streptococcus pneumoniae* STRAINS RESISTANT TO BETA-LACTAM ANTIBIOTICS

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SUMMARY

Streptococcus pneumoniae is a major pathogen that causes Otitis Media infections and bacterial meningitis in children as well as community acquired pneumonia in adults. Clinical isolates of *S. pneumoniae* exhibiting resistance to β-lactam antibiotics are being isolated with increased frequency in many countries. Altered penicillin-binding proteins is the main mechanism of resistance that *S. pneumoniae* has acquired. The acquisition of this resistance mechanism has been attributed to horizontal gene transfer mediated especially by the natural transformability of *S. pneumoniae.* Other mechanisms of resistance to β-lactam antibiotics in *S. pneumoniae* are less known.

This study was focused on *S. pneumoniae* and its increased level of resistance to β-lactam antibiotics. Eleven clinical isolates from Duke University in Durham NC, Grady hospital in Atlanta, GA and one from Boston, MA Hospital were used in the first phase of my research. In addition, this study included six strains obtained from the Centers for Disease Control and Prevention (CDC), Atlanta, GA., which were used in the formulation of one widely used pneumococcal polysaccharide vaccine. All strains were tested for suseptibility to amoxicillin, ampicillin, ceftazidime and vancomycin. Representative strains were also tested for susceptibility to penicillin G. All of the clinical isolates exhibited high levels of resistance to at least one antibiotic. One strain in particular, Sp D2 was resistant to all of the β-lactams tested, but was susceptible to vancomycin, the only non-β-lactam used in the first phase. These strains were also characterized by SDS-PAGE to compare their whole-cell protein profiles. The proteins in the highly resistant strain (Sp D2) exhibited a unique SDS-PAGE profile with one especially highly-resolved band with a molecular weight of approximately 50 kDa that was not present in any

other strain. Additional SDS-PAGE analysis also revealed that the Sp D2 protein band of interest may represent two proteins.

The unique band exhibited by Sp D2 was further characterized by membrane protein extraction, followed by In-Gel Trypsin digests. The resulting peptide digests were analyzed further using MALDI-MS (matrix assisted laser desorption and ionization mass spectrometry). Spectra obtained from the tandem mass spectrometry analysis were used to search databases for peptide sequence homologies. The resulting MALDI-MS data did not produce any significant matches with proteins in any of *S. pneumoniae* published genome databases. In addition, N-terminal and Internal sequencing performed at Emory's Microchemical Facility did not result in any significant matches; thus, indicating the possibility of a new protein.

We consultation with Dr. Keith Klugman a Professor in the Infectious Diseases/ International Health Department at the Emory School of Public health and Dr. Richard Facklam of the Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, CDC, about my initial thesis results. Both individuals are members of the worldwide Active Bacterial Core Surveillance/Emerging Infections Program Network. They expressed immediate interest in the results I had obtained and stressed that the unique characteristics exhibited by Sp D2 could be of great importance and of international significance. They informed us that Sp D2 exhibited levels of resistance to β-lactam antibiotics rarely documented. The constant monitoring of drug resistant strains are essential in planning for effective treatment and/or vaccine development.

We therefore initiated collaboration with Dr. Facklam and the Active Bacterial Core Surveillance/Emerging Infections Program Network at CDC to further study Sp D2 and expanded my research to include the other clinical isolates presented in my thesis.

Phenotypic characterization of strains is not always reliable since they are influenced by environmental changes such as pH, temp etc. It was therefore necessary to confirm that Sp D2 was in fact a *Streptococcus pneumoniae*. This was especially important since the protein study results did not receive a match with the three sequenced *S. pneumoniae* genomes. This second phase of my research included serotyping (capsule based), extensive susceptibility testing as well as PCR studies targeting genes that conclusively identify *S. pneumoniae* and that encode proteins being considered as candidates for new vaccine development.

Sp D2 was found to be non-typeable and therefore could not be assigned a capsule type. This is unusual since 99% of *S. pneumoniae* strains are typeable. Which means that the current capsular polysaccharide vaccine would not be active against an Sp D2 infection. Additional biochemical tests by bile solubility and optochin tests showed Sp D2 to be positive for both tests, a characteristic of most *S. pneumoniae* strains. PCR of the pneumococcal surface protein gene (*psaA*), Autolysin gene (*lytA*) and pneumolysin gene (*ply*) showed that Sp D2 contained all three genes. Sp D2 was therefore positively identified as a *S. pneumoniae* strain, though non-typeable. Two other clinical isolates Sp G-5481 and Sp Original were also non-typeable. All the other clinical isolates were positive for p*sA* and *lytA* by PCR. Two strains, Sp G-31159 and Sp G-5481 were negative for the presence of p*ly*. Both strains still identified as fitting the profile of *S. pneumoniae*.

Additional antibiotic resistant tests on Sp D2 revealed resistance all β-lactams tested, including cephalosporins. This means that Sp D2 infections could be treated with any β-lactam antibiotic. It is also resistance to erythromycin which is a macrolide. Sp D2 was found to be susceptible to four antibiotics, chloramphenical, tetracycline,

clindamycin (a macrolide), levofloxacin and vancomycin. However, tetracycline, clindamycin and levofloxacin are not suitable for treatment of meningitis. This leaves only chloramphenical and vancomycin. Unfortunately, vancomycin must be administered intravenously to treat pneumococcal infections and its use tends to select for vancomycin-resistant Enterococci. The fluoroquinolones have the drawbacks of being quite broad in spectrum and relatively expensive. Thus an infection or outbreak caused by Sp D2 would present a serious treatment problem.

CHAPTER I

INTRODUCTION AND BACKGROUND

Taxonomy of *S. pneumoniae*

Streptococcus pneumoniae is an important bacterial pathogen worldwide. This organism is a member of the normal microflora of healthy children and adults, but can sometimes cause infections. *S. pneumoniae* can cause invasive infections such as pneumonia, meningitis, and septicemia, as well as localized infection in children known as Otitis Media (2, 36, 56, 77). Acute Otitis Media is one of the major causes of morbidity in children (30). The most severe forms of Otitis Media is caused by three pathogens, *S. pneumoniae, Moraxella catarrhalis,* and *Haemophilus influenzae.* The focus of my research is only on *S. pneumoniae*.

Otitis media is an infection of the middle ear that occurs when the Eustachian tube fails to properly drain fluid (30). The Eustachian tube in infants is shorter, wider and more horizontal than in older children and adults. Also, the cartilage supporting the Eustachian tube in infants is floppy, and cannot properly drain the middle ear fluid. Consequently, infection occurs when bacteria migrate from the nasopharynx into the Eustachian tube and proliferate, therefore activating the inflammatory response resulting in tissue damage and Otitis Media.

Treatment and management of Otitis Media infections caused by *S. pneumoniae* currently involve chemotherapy with antimicrobial agents, and/or immunizations with the pneumococcal polysaccharide vaccines. Several different groups of antimicrobial agents

are used by physicians to treat patients. Knowledge of the causative agents, and their antimicrobial susceptibility patterns are essential to guide the appropriate selection of antimicrobial agents to use in chemotherapy.

When evaluating taxonomic information on clinical isolates, scientists and physicians must consider three broad categories of fundamental biological information that pertain to bacterial pathogens: (i) criteria used to definitively classify a clinical isolate into a Genus and species, (ii) intra-species differentiation such as serotypes, resulting from strain-to-strain variation, and (iii) variation in expression and regulation of genes that encode virulence factors.

Phenotypic Characteristics of *S. pneumoniae*

Since *S. pneumoniae* is an important pathogen, correct identification and characterization is critical. For routine clinical laboratories, identification of pneumococci has been determined on the basis of several phenotypic characterization methods (2, 36, 62).

S. pneumoniae is a gram positive organism that is identified by using several phenotypic tests including selective and differential growth media. When cultivated on blood agar plates, this organism exhibits a characteristic greenish halo around colonies. This is alpha hemolysis resulting from partial lysis of red blood cells. Other *Streptococci* such as *S. pyogenes* exhibit beta hemolysis which is total red blood cell lysis. The bile solubility test is yet another method used to identify *S. pneumoniae*. The organism produces an enzyme called amidase that cleaves specific covalent bonds in the peptidoglycan layer. This enzyme is activated by bile or bile salts such as sodium

deoxycholate. Another test is the Optochin test which is a presumptive test that is used to identify strains of *S. pneumoniae*. Optochin is a surface-active agent that specifically interacts with the cell membrane to produce cell lysis (2). Three phenotypic tests (optochin susceptibility, bile solubility, and agglutination with the antipneumococcal polysaccharide capsule antibodies) were previously used to differentiate classical *S. pneumoniae* from other alpha hemolysis *Streptococci* (90).

Antimicrobial Susceptibility Testing

Following presumptive identification, it is necessary to inform physicians of susceptibility patterns of the organisms. In a clinical setting, *S. pneumoniae* susceptibility patterns are routinely obtained. This is especially important in that it helps in selection of antibiotics to use for treatment. The agents routinely used to treat *S. pneumoniae* infections are discussed in more detail below (page 12).

Other Specialized Tests for Characterization of *S. pneumoniae*

While phenotypic characterization is useful for convenience, they are however not always reliable. This is because phenotypic properties are influenced by many environmental factors such as pH, temperature, age of culture etc. To provide more definitive information for proper identification of organisms, molecular techniques are being used especially in epidemiological studies of outbreaks. Several categories of molecular biological assays are used.

Some immunological based tests include antibody assays to detect specific pathogens. This can also be used to differentiate between strains of the same species.

ELISA (Enzyme-linked immunosorbent assay) measures antibodies to whole cells and/or cellular components. Western blot analysis allows one to visualize antibodies directed against a specific protein usually on an SDS-PAGE gel. The immunological based tests are highly accurate however their main disadvantages include expense and time involved in raising antibodies. They also require reagents in which all serotypes are represented (61).

SDS-PAGE analysis can be used to for whole cells protein analysis or analysis of specific fractions such as cytoplasmic membranes and outer membranes of gram-negative bacteria. This is an important molecular epidemiological tool because it generates a profile that can distinguish between strains that otherwise may have very similar characteristics based on phenotypic and biochemical tests (76). This is especially important in identification of atypical strains.

Some other molecular based tests that can be used to identify *S. pneumoniae* include nucleic acid hybridization and PCR to detect specific characteristic genes. The current PCR techniques are especially useful due to the accuracy and speed involved in obtaining results.

Virulence Factors of *S. pneumoniae*

Capsule

S. pneumoniae encapsulated strains have been shown to be more virulent than those without a capsule. The study of pneumococcal capsule was the object of many investigations that led to important scientific discoveries. In 1928, Griffith showed that when heat-killed encapsulated pneumococci and live strains lacking a capsule were

together injected into mice, the none capsulated strain could be converted into encapsulated pneumococci and kill the mice. Years later, the carrier of this genetic information was shown to be DNA (2).

Serotyping of *S. pneumoniae* is important because there have been changes in the distribution of types isolated from invasive diseases over a period of time in a single locality. This is important with reference to vaccine formulations. Differences in the capsular types that are isolated from different age groups and regional differences in distribution of types have also been reported (44).

The capsule has long been recognized as the major virulence factor of *S. pneumoniae*. The chemical structure of the capsular polysaccharide and, to a lesser extent, the thickness of the capsule determine the differential ability of the strains to survive in the bloodstream and possibly cause invasive disease (2). The capsule prevents binding of antibodies to the cell wall of pneumococci and thus inhibits phagocytosis.

Pneumococci can be divided into 90 serotypes on the basis of difference in capsular polysaccharide structure but most cases of invasive infections appear to be caused by twenty three serotypes (2, 35). The currently licensed 23-valent polysaccharide pnemococcal vaccine which is only moderately effective contains twenty three purified capsular polysaccharide antigens; serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F (36). The serotypes 6B, 9V, 14, 19A, 19F, and 23F cause most drug-resistant infections in the United States (36).

The distribution of types isolated from adults differs substantially from that of types isolated from children. Some studies have shown that the most important pediatric serotypes, 6A, 14, 19F and 23F were responsible for almost 60% of all infections. In

adults however, serotypes 3, 19F and 6A accounted for only 31% of the isolates (2). The geographical distribution and prevalence of serotypes differ worldwide. Twenty serotypes are responsible for ~ 90% of all reported infections in the United Sates and Europe, whereas, for instance, the 23-valent vaccine, which contains these serotypes is effective against <70% of pneumococcal infections in Asia (2). In 2000, a 7-valent protein-polysaccharide conjugate vaccine targeting seven pneumococcal serotypes was licensed in the Unites States for use in young children (92).

Pneumococcal Surface Antigen

PsaA gene in *S. pneumoniae* encodes a protein which functions to transport Mn^{2+} and Zn^{2+} into the cytoplasm (36, 51). Pneumococcal surface antigen A (PsaA) is a virulence factor of pneumococci and has a molecular weight of approximately 37 kDa (37, 61). The absolute determination of PsaA specificity for Mn^{2+} and/or Zn^{2+} still remains to be answered. However, growth requirements of $PsaA^-$ mutants suggest that PsaA plays an essential role in the transport of Mn^{2+} into the cytoplasm of pneumococci (36). Mutations in *psaA* result in growth limitation in low manganese(51). PsaA elicits protective properties in mice against *S. pneumoniae* and the $PsaA^-$ mutants of pneumococci were also avirulent in a mouse model (36). Marra et al examined the expression of *psa* operon *in vivo* and the virulence of deletion mutants were assessed in different animal models of infection. The results demonstrated that *psaA* mutants were completely attenuated in systemic, respiratory tract and Otitis media infections. In addition, the mutants were unable to grow in an implanted peritoneal chamber, but growth was restored by the addition of manganese to the chambers (51)

There is still a debate on whether PsaA protein plays a role in pneumococcal adhesion. Some investigations of PsaA have suggested that this molecule is not an adhesin, as initially thought. The lipid-linked PsaA protein, with dimensions of 40 by 40 by 70 Å, is probably present beneath the peptidoglycan and capsule structures of pneumococci (36). The total thickness of the pneumococcal cell wall is approximately ~0.36 μm, therefore PsA has no possibility of protruding outside the cell wall (36). Other studies involving mutation analysis demonstrated that changes in PsaA protein affect the binding of *S. pneumoniae*, indicating that PsaA plays a critical role in bacterial adherence and virulence (37).

Morrison et al demonstrated the presence of *psaA* in all 90 serotypes that were tested, and that there was lack of amplification of heterologous organisms. This suggested that PCR amplification of *psaA* could be a very useful tool for detection of pneumococci and diagnosis of disease. This study showed that a protein common to all serotypes with genetic and immunologic similarity has implications for both vaccine studies and diagnostics development. It meant that the use of an immunogenic common protein as a vaccine could eliminate the need for multiple capsular types in a pneumococcal vaccine and additionally elicit a memory response, which occurs only with protein-based vaccines (61).

Genomic sequence analysis shows that PsaA belongs to an ATP binding cassette (ABC)-type transport system and constitutes the extracellular component responsible for solute (metal) binding (36). The ABC-type transport system is characteristic of prokaryotic and eukaryotic cell and has several domains. The individual domains can be expressed as separate proteins or may be fused into multidomain polypeptides in variety

of ways (68). It has an extra-cytoplasmic protein responsible for solute binding (such as PsaA), an integral membrane part responsible for transport of the solute through the cell membrane, and a cytoplasmic protein binding ATP. ATP binding cassette (ABC)-type multidrug transporters use the free energy of ATP hydrolysis to pump drugs out of the cell, the so called efflux pumps (68). This has been demonstrated in several bacteria. Fluoroquinolone resistance in pneumococci is known to be associated with the efflux pump(7).

Pneumolysin

Pneumolysin (Ply) is a virulence factor of pneumococci that penetrates the physical defenses of the host. It is a 53-kDa protein produced by most clinical isolates of the pathogen (36). Unlike other pneumococcal antigens, this molecule is not surface exposed. It is a cytoplasmic enzyme that is released due to the action of the surface pneumococcal autolysin (2, 36). The enzyme is cytotoxic to ciliated bronchial epithelial cells and slows beating in organ culture (2).

Pneumolysin is part of a larger group of proteins of pathogenic gram-positive bacteria known as cholesterol-dependent cytolysins. All of them are virulence factors for their organisms (36). Their mode of action is based on binding to the host cytoplasmic membrane cholesterol, a process that is followed by insertion into the targeted membranes and formation of relatively large pores. Once the pores are formed, the targeted cells cell undergoes lysis (36).

Autolysin

Autolysins are members of a widely distributed group of enzymes that degrade the peptidoglycan backbone of bacteria (36). The action of these cell wall degrading enzymes ultimately lead to cell lysis. An example of one such enzyme is the *S. pneumoniae* N-acetylmuramoyl-L-alanine amidase, also known as LytA amidase. LytA has a molecular mass of ~ 36 kDa and has been implicated in the pathogenesis of pneumococci. One of the direct implications is the release of the components of cell wall shown to be highly inflammatory in animals (36). The indirect implication involves the release of cytoplasmic bacterial proteins including pneumolysin discussed above.

The precise role of LytA in virulence is still however under debate. Berry et al, demonstrated that mutations of *LytA* gene in the *S. pneumoniae* chromosomes leads to significantly decreased virulence of this organism compared to wild-type strain in mouse intraperitoneal challenge (6). Other studies of LytA also have shown that this amidase induces a protective response in mice to streptococci when inoculated in the lungs (11). This protective property of LytA that contributed to a significantly longer survival for mice challenged intranasally with autolysin makes it a potential component of novel anti-pneumococcal vaccines (36).

Alternative Approaches to Vaccine Development and Diagnosis of *S. pneumoniae*

(Importance of PsaA, Ply and LytA study)

As discussed earlier, pneumococci can be divided into ~90 serotypes on the basis of difference in capsular polysaccharide structure but most cases of invasive infections appear to be caused by twenty three serotypes (2, 35). The distribution of types isolated

from adults differs substantially from that of types isolated from children. The geographical distribution and prevalence of serotypes differ worldwide. Twenty serotypes are responsible for ~ 90% of all reported infections in the United Sates and Europe, whereas, for instance, the 23-valent vaccine, which contains these serotypes is effective against <70% of pneumococcal infections in Asia (2).

The existence of many pneumococcal serotypes has complicated further development of the current capsular polysaccharide classes of vaccines. Although these vaccines cover the most prevalent serotypes found in the population at risk, they protect mainly against illness caused by those serotypes included in the vaccine. Further more, the current 23-valent polysaccharide vaccine poorly induces antibody responses in young children under the age of two years old and thus does not effectively protect against either invasive or local pneumococcal infections (77). The problems with the current vaccine has stimulated an interest in the need for new approaches to pneumococcal vaccine development and consideration of other cellular components as vaccines.

Considerable research is being conducted on potential third-generation common protein vaccines. Candidates under consideration include PsaA and a recombinant nontoxic pneumolysin (Ply) amongst others (37, 77). These pneumococcal proteins, which are T cell dependent, appear to be common to all pneumococcal serotypes and are likely to be highly immunogenic in humans and to elicit immunologic memory (37). In contrast to current pneumococcal vaccines, third-generation vaccine candidates will provide protection to a broader target population, from infants to elderly individuals and those at high risk. Furthermore, it is hoped that these will not be geographically specific and will provide protection against all pneumococcal serotypes. Another advantage is that

the new vaccines could be produced in large quantities at low cost with recombinant technology in an appropriate host such as *E.coli* (77).

The increase in pneumococcal antibiotic resistance has further complicated the problem. The serotypes 6B, 9V, 14, 19A, 19F, and 23F cause most drug-resistant infections in the United States (36). In some areas, 35%-50% of pneumococcal isolates are resistant to penicillin. Many of these isolates are also resistant to other antimicrobial drugs, with some isolates susceptible only to vancomycin. Recently though, vancomycin tolerance emerged in pneumococci (36). This reinforces the need for an effective improved vaccine and/or effective new drugs against pneumococcal infections.

Although *S. pneumoniae* is the most common bacterial cause of pneumonia in children and adults worldwide, the diagnosis of pneumococcal pneumonia is difficult to establish. Blood cultures are positive in only 20%-30% of adults and less than 10% in children with pneumococcal pneumonia (10, 70). Sputum is also difficult to obtain and young children do not produce sputum. Further more, sampling via bronchioscope is laborious.

Other specialized tests for characterization of *S. pneumoniae* include PCR. The genes that have been targeted are the genes encoding pneumolysin, autolysin, and pneumococcal surface protein (69). Salo et al, initially developed a PCR assay for the etiologic diagnosis of pneumococcal pneumonia. They targeted the pneumolysin gene and used nested PCR to amplify a 348-bp and a 208-bp fragment of the gene (70). Nested PCR means that two pairs of PCR primers were used for a single locus. The first pair amplifies the locus as seen in any PCR experiment. The second pair of primers (nested primers) bind within the first PCR product and results in a second PCR product that will

be shorter than the first one. The logic behind this strategy is that if the wrong locus were amplified by mistake, the probability is very low that it would also be amplified a second time by a second pair of primers. The initial PCR assay developed by Salo et al was prone to contamination problem and was further modified. The modification was based on the amplification of a 209-bp segment of the pneumolysin gene. For demonstration of the amplification product, microwell hybridization with a Europium-labeled oligonucleotide probe complementary to a biotinylated strand of the PCR product was performed, and the presence of the PCR product was monitored by time-resolved fluorescence of the Europium chelate (69). This modified assay proved to be reliable and is suitable for automation therefore can be used to test high numbers of samples.

Several PCR methods for identification of *S. pneumonia* have been developed by Dr. Facklam's group at the CDC (37, 54, 55, 61). They targeted the gene encoding pneumococcal surface antigen adhesin A (PsaA). An 838-bp fragment of the gene was amplified in all strains tested. This PCR assay has by far been the most powerful assay developed for detection of *S. pneumoniae* for several reasons. First, they demonstration of *psaA* in all pneumococcal serotypes . This has clinical significance because of the potential to design assays to detect all *S. pneumoniae* isolates regardless of serotype. Second, this Assay also showed lack of amplification of heterologous organisms suggesting that this could be a useful tool for detection of pneumococci in clinical samples (61).

Chemotherapy and Management of *S. pneumoniae* Infections

Treatment and management of *S. pneumoniae* infections currently focus on antimicrobial agents and vaccines (described above). Several different groups of antimicrobial agents are used to treat *S. pneumoniae* infections. By far the most common agents are the β-lactam antibiotics which include Penicillins, Cephalosporins (Figure 1), Monobactams and Carbapenems. β-lactams act by inhibiting bacterial cell wall synthesis. Vancomycin though not considered a β-lactam antibiotic, also prevents cell wall synthesis. Another group of antimicrobial agents used to treat *S. pneumoniae* infections are Macrolides such as Erythromycin. These act by binding free ribosomes thus preventing protein synthesis. Sulfonamides such as sulfamethazole act by preventing the synthesis of bacterial folic acid (31). Fluoroquinolones are also used, and these bind to DNA gyrase, thus preventing bacterial DNA synthesis.

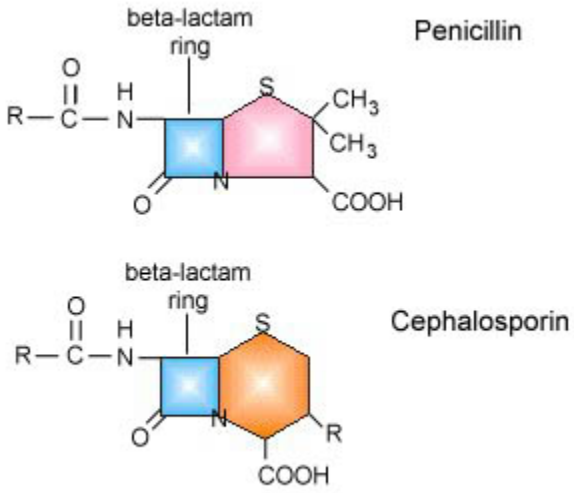

Figure 1: Diagram of Penicillin and Cephalosporin, modified from (67)

The β-lactams are by far the most widely used and efficacious of all antibiotics. The current choice of treatment of pneumococcal infections are β-lactams such as, amoxicillin (a penicillin derivative). β-lactam antibiotics prevent cell wall synthesis by targeting transpeptidases (33).

The cell wall of bacteria is a peptidoglycan (murein) layer composed of two sugar derivatives, (N-acetylglucosamine and N-acetylmuramic acid) and a small group of amino acids (Figure 2). Synthesis of peptidoglycan during cell growth involves controlled cutting by autolysins of bonds connecting small areas of preexisting peptidoglycan, with the simultaneous insertion of new pieces of peptidoglycan. The final step in cell wall synthesis is formation of the peptide cross-links between adjacent glycan chains. This is the step catalyzed by transpeptidase (also known as penicillin-binding proteins). Inhibition of the transpeptidation step by β-lactam antibiotics thus leads to the formation of weakened peptidoglycan which eventually leads to lysis of the bacterial cell (96).

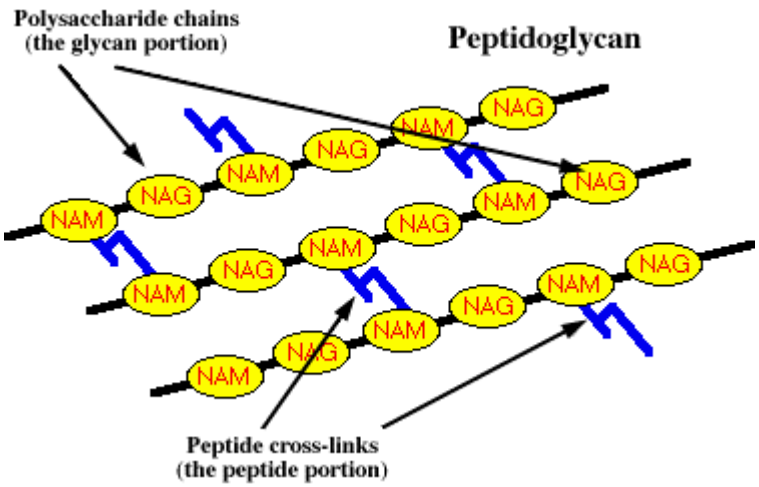

Figure 2: Schematic Section of Bacterial Peptidogylcan layer, modified from (67)

Antibiotic Resistance

Antibiotic resistance has become a disturbing problem in the medical community. For many years after the introduction of penicillin, commonly encountered gram-positive bacterial pathogens were susceptible to this and other relatively simple antibiotics. However, spontaneous mutation or the acquisition of genetic determinants conferring antibiotic resistance and selection of these resistant bacterial strains occurred fairly quickly (46). *S. pneumoniae* was formerly uniformly susceptible to penicillin, but as early as 1960, strains resistant to penicillin were reported. Unfortunately, penicillin-resistant pneumococci tend to be resistant to other antibiotic classes as well, notably the sulfonamides, tetracyclines, macrolides, clindamycin and chloramphenicol (46).

Currently, the most effective antibiotics against penicillin-resistant pneumococcal strains are vancomycin and certain of the newest fluoroquinolones. However, vancomycin must be administered intravenously to treat pneumococcal infections and its use tends to select for vancomycin-resistant Enterococci. The fluoroquinolones have drawbacks due to the ease of acquiring resistance by changing only one nucleotide in the DNA gyrase and relatively expensive (46). Thus, the evolution of frequent, high-level penicillin resistance in the pneumococcus has led to a reappraisal of the treatment of common respiratory tract infections. This includes a greater emphasis on routine susceptibility testing of pneumococcal clinical isolates, increased efforts to detect resistance in pneumococci, and use of the recently developed protein-polysaccharide conjugate vaccine in prevention of clinical infections (46, 92).

Antibiotic Resistance Mechanisms: β-Lactamases

Among Otitis media pathogens, the simplest mechanism of resistance is the production of β-lactamases (27). β-Lactamases are a group of microbial enzymes belonging to several classes that all function by cleaving the β-lactam ring of these antibiotics, thus inactivating them. As these enzymes are frequently plasmid encoded, resistance can readily be transmitted between bacteria. *S. pneumoniae* is naturally competent for genetic transformation. This competence in *S. pneumoniae* is regulated by a quorum-sensing system (45). Despite the fact that pneumococci are naturally transformable, current researchers state that no β-lactamase producing strain has been described (27). Microorganisms such *Haemophilus influenzae* and *Moraxella catarrhalis* in combination with *S. pneumoniae* cause respiratory tract infections and Otitis Media; yet both of those strains possess β-lactamases. Since *S. pneumoniae* is a natural transformant, it would seem probable that they would also possess β-lactamase genes. This however has not been widely studied. Due to the absence of β-lactamases, penicillin-resistant pneumococci are not susceptible to combination drugs such as amoxicillin-clavulanate and ampicillin-sulbactam which contain β-lactamase inhibitors (46).

Antibiotic Resistance Mechanisms: Altered Penicillin-Binding Proteins

Evidence from studies on laboratory isolates of penicillin-resistant pneumococci and on the genetic transformation of resistance has indicated that penicillin resistance is a multigenic property acquired in a stepwise fashion (28). It was established that resistance is associated with alterations in the penicillin –binding proteins (PBPs) that catalyze the

terminal steps in the assembly of the bacterial cell wall and are the targets of β-lactam antibiotics (28).

Early studies using radioactively labeled penicillins incubated with isolated bacterial membranes subjected to SDS-PAGE analysis showed that *S. pneumoniae* had 3 major groups of penicillin-binding proteins named PBPs 1, 2, and 3 (12, 65). Comparisons of susceptible strains and resistant strains revealed that resistant bacteria retain a normal set of PBPs, but one or more of these appear to have lower binding capacity for the antibiotic than the same PBPs from susceptible cells (26, 65). In order to establish the nature and properties of these PBPs, studies were conducted with the proteolysis of the PBPs. Results indicated that the β-lactam binding sites of the PBPs were similar. The PBPs were also converted into soluble, hydrophilic derivates indicating that they were membrane bound (21). Immunological studies using antibodies prepared against the penicillin-susceptible PBPs indicated that the resistant strains had PBPs that were immunologically related to the susceptible PBPs with the difference being only in the binding affinity to β-lactams (26).

To date it has been established that *S. pneumoniae* contain six PBPs which are classified into two group mainly based on their molecular sizes (96). Group I consists of the low-molecular-mass PBPs. These exhibit DD -Carboxypeptidase activity, removing the C-terminal D-alanine of the pentapeptide side chain in the murein (71). They appear to be important but non-essential for cell growth (42, 96). In *S. pneumoniae* this is represented by PBP3 (43 kDa). This protein has been proposed to be dispensable, since a variety of β-lactams are bound by PBP3 far below their respective MICs and since the

penicillin affinity of PBP3 is not altered in intrinsically penicillin-resistant strains (42, 71).

Group II PBPs are the high-molecular-mass PBPS. Unlike the low-molecular-mass PBPs, these are essential (96). These possess essential transpeptidase (cross-linking) and transglycosylase (elongation) activities responsible for bacterial cell wall peptidoglycan synthesis (33). The transpeptidase activity of these PBPs is inhibited by β-lactam antibiotics.

Table 1: Summary of *S. pneumoniae* Penicillin-Binding Proteins

PBP	Molecular Mass	Importance in Resistance Development
*PBP 1a	98 kDa	Required for high level β-lactam resistance.
PBP 1b	89 kDa	Role not established
*PBP 2x	82 kDa	Primary resistance determinants and confer low- level β-lactam resistance. Prerequisite for high resistance
PBP 2a	81 kDa	Some low affinity forms have been found
*PBP 2b	77 kDa	Primary resistance determinants and confer low- level β-lactam resistance. But does not interact with expanded-spectrum cephalosporins.
PBP 3	43 kDa	Does not participate in resistance development

* PBPs found to be altered in high level resistant isolates. The above table is summarized from Hakenbeck et al (26, 27) and Laible et al (43).

The high-molecular-mass PBPs have been subdivided into class A and class B, which differ in part by sequences of the N-terminal regions (33). In *S. pneumoniae*, class A PBPs are represented by PBP1a (98 kDa), PBP1b (89kDa), and PBP2a (81kDa), and

class B PBPs are represented by PBP2x (82 kDa) and PBP2b (77 kDa) (21, 33). The C-terminal domains of both classes appear to possess transpeptidase activity. The functions of the N-terminal domains are less established, but for class A high-molecular-mass PBPs evidence suggests that they possess transglycosylase activity (33). The N-terminal domains of the class A PBPs contain four conserved motifs that are also present in monofunctional transglycocylases, and several of the class A proteins have been shown to catalyze the polymerization of the polysaccharide backbone of peptidoglycan in vitro (33).

The structure of a penicillin-binding protein, a soluble derivative of *S. pneumoniae* PBP2x, has recently been determined by X-ray crystallography (63). It consists of three domains: an N-terminal domain with the appearance of a 'sugar-tongue' that is in close association with a second, central penicillin-binding domain, and a C-terminal domain that is connected via 28-residue-long flexible loop. The penicillin-binding domain which is homologous to that of other PBPs and β-lactamases, contains three conserved amino acid motifs that are in a close spatial relationship as part of the active site of the enzyme (27). PBPs interact with β-lactams by forming a relatively stable covalent complex via the active site serine nucleophile. Eventually, deacylation of this complex takes place, resulting in a biologically inactive β-lactam derivative.

Mutations that contribute to resistance are located in the transpeptidase-penicillin binding domain and they reduce the affinity to β-lactams rather than affecting the deacylation step (27). Several mutations in one PBP may be required to cause a substantial decrease in penicillin-binding affinity, and more than one PBP may have to be changed into low-affinity variants in order to achieve high resistance levels. In *S.*

pneumoniae, alterations in the PBP1a, PBP2x, and PBP2b confer low resistance and are the prerequisite for high levels of resistance mediated by mutations in other PBPs (25, 50).To date, sequences of *S. pneumoniae* PBPs from several susceptible and resistant strains are known and are available through GeneBank.

There has been extensive research done on *S. pneumoniae* PBPs but there are still however some areas that need more studies. For example, the role of some of the high-molecular-mass PBPs such as PBP1b and PBP2a is vague. It has also been established that the low-molecular-mass PBP3 does not play a role in resistance development. However there have been occasions where resistant strains have been isolated that exhibited a higher than normal molecular weight but the reasons behind these observations have not been fully explored. PBP2x has also been shown to be a prerequisite for high resistance to β-lactams. It has not yet however been determined which of the many substitutions in the PBP2x gene contribute to the greatly decreased affinity of PBP2x for β-lactams (43).

Recently, a third level of resistance mechanisms has been identified in laboratory mutants, wherein non-PBP genes are mutated and resistance development is accompanied by deficiency in genetic transformation (27). Two such non-PBP genes have been described: a putative glycosyltransferase, CpoA, and a histidine protein kinase, CiaH. It has been proposed that these non-PBP genes are involved in the biosynthesis of cell wall components at a step prior to the biosynthetic functions of PBPs, and that the mutations selected during β-lactam treatment counteract the effects caused by the inhibition of penicillin-binding proteins (27). This mechanism of resistance is however still under investigation.

Other mechanisms of resistance directed towards non-ß-lactam drugs used to treat *S. pneumoniae* infections may include production of adenine dimethylase, which acts on the 23S ribosomal RNA to reduce macrolide binding to the ribosome (31). Resistance to sulfonamides may involve mutations in target enzymes responsible for folic acid biosynthesis, and resistance to Fluoroquinolones may also involve mutations in DNA gyrase genes, preventing binding to DNA gyrase (31).

The Role of Horizontal Gene Transfer in Development of *S. pneumoniae* Altered Penicillin-Binding Proteins

Gene transfer among bacteria may occur by either of three processes: (i) Conjugation, a process that requires cell-to-cell contact, (ii) Transduction, a process that is mediated by a bacteriophage, and (iii) Transformation, a process in which a recipient cell acquires plasmids or free (extracellular) fragments of DNA from its surroundings, and subsequently incorporates such fragments into its chromosomal DNA.

The mechanism of natural genetic transformation by bacteria is described in a review by Lorenz and Wackernagel (48). These authors describe natural gene transformation to distinguish it from *in vitro* (artificial) procedures used to introduce DNA molecules into bacterial cells. Bacteria are the only organisms capable of natural genetic transformation. *S. pneumoniae* is historically known for its ability to carry out the process of natural gene transformation. The process of natural gene transformation only occurs during a specific period in the exponential growth phase when cells are in a state of competence. The state of competence develops suddenly in response to cell-to-cell signals, and its duration is approximately 20 minutes. Lee and Morrison (45, 89)

identified several genes in *S. pneumoniae* that regulated competence, and linked quorum sensing to the development of competence for natural genetic transformation.

Naturally transformable organisms such as *S. pneumoniae* have ready access to a wide pool of genetic diversity from closely related species, and it is the frequency with which advantageous genes can be shuffled that has resulted in these organisms becoming resistant to antibiotics in a period of less than 30 years (20). Perhaps the best recorded examples of mosaic genes related to antibiotic resistance that have arisen from interspecies recombination are the altered penicillin-binding proteins (PBPs) in *Streptococcus* and *Neisseria* (50). The large-scale use of antibiotics has created a selection pressure that has promoted the spread of mosaic genes encoding proteins with decreased affinity for at least two important classes of antimicrobial agents: the ß-lactams and the sulfonamides.

Genetic analysis of genes encoding PBPs concluded that these mosaic genes were the result of localized recombination events between *S. pneumoniae* and the homologous genes from *Streptococcus mitis* and S*treptococcus oralis* (20). These organisms are commensal members of the oral flora. As these bacteria coexist within the oral cavity it is quite feasible that there is a complex network of interspecies gene transfer (90). It has been shown that to be resistant to penicillins, a pneumococcus must acquire low-affinity variants of three PBPs (PBPs 1a, 2b, and 2x). The accumulation of three separate penicillin-resistant PBPs encoded by mosaic genes seems at first sight unlikely, but nucleotide sequence data show that this has occurred on many separate occasions, leading to the widespread penicillin resistance observed in the pneumococcus. Moreover, in *S.*

pneumoniae pbp1a and *pbp2x* are cotransformable, by virtue of their proximity on the chromosome. This resistance hence has the potential for rapid spread among strains (50).

Genetic studies comparing sequences of *pbp2x* in some susceptible and resistant strains strongly suggests that the altered PBP2x genes have arisen by localized interspecies recombinational events. One particular study by Laible et al (43), showed that in a 2kb region, the susceptible *S. pneumoniae* clinical isolates differed at only 8 nucleotide sites (0.4%) and resulted in one single amino acid difference in PBP2x. In contrast, the sequences of the PBP2x genes from the resistant isolates differed overall from those of the susceptible isolates between 7 and 18% of nucleotide sites and resulted in between 27 and 86 amino acid substitutions in PBP2x. The altered PBP2x genes consisted of regions that were similar to those of susceptible strains (< 3% diverged), alternating with regions that were very different (18-23% diverged). The presence of highly diverged regions within the PBP 2x genes of the resistant isolates contrasted with the uniformity of the sequences of the amylomaltase genes from the same isolates, and with the uniformity of the PBP2x genes in the susceptible isolates.

More recently, the emergence of multidrug-resistant *S. pneumoniae* variants are thought to arise through natural transformation involving recombinational replacements, within and around the capsule biosynthesis (*cps*) locus, of DNA fragments sometimes as large as 25 kb (17). The *cps* locus of *S. pneumoniae* is flanked by the *pbp2x* and *pbp1a* genes coding for penicillin-binding proteins, enzymes involved in the cell wall synthesis that are targets for β-lactam drugs (85).

Natural Transformation in *S. pneumoniae*

The term "natural genetic transformation" has been coined to distinguish it from other (artificial) in vitro procedures used to introduce DNA molecules into bacterial cells (48).

The first evidence of bacterial transformation was obtained by British scientist Fred Griffith in the late 1920s. Griffith was working with *S. pneumoniae*, a bacteria that invades the body with the help of a polysaccharide capsule. Griffith discovered that isolates lacking the capsule (formed R- rough colonies) were unable to cause infection when injected to a mouse. A mouse infected with the capsule strain (formed S- smooth colonies) would cause infection in a day or two. Griffith showed that if heat killed S cells were injected along with R cells, there was a fatal infection, and the bacteria isolated from the dead mouse were S type. This meant that the R cells had been transformed to a new type. The molecular explanation for the transformation was later provided by Oswald T. Avery and colleagues in the 1930s. They were able to conduct the same transformation in a test tube. After fractionating, they purified the active fraction of cell-free extracts and showed it to consist of DNA.

Competence for transformation in Streptococci is not constitutive, as it is in Neisseria species, but is regulated by a quorum-sensing system encoded by two genetic loci, comCDE and comAB (45, 89). The *com* operon contains three genes *comC, comD* and *ComE*, encoding a competence-stimulating peptide (CSP), histidine kinase, and a response regulator respectively (45, 89). Two genes located elsewhere on the chromosome, *comA* and *comB*, encode proteins responsible for the export of CSP from the cell (89). CSP induces competence when a critical extracellular concentration is

reached. The *comD*-encoded transmembrane histidine kinase is believed to be a receptor for CSP and to phosphorylate a *comE*-encoded transcription regulator, producing an active form that up regulates both the *comCDE* operon and, a number of other genes involved in competence development (45, 89).

Tomasz and Hotchkiss were two pioneers that paved the way to understanding natural transformation in *S. pneumoniae.* Their studies involved the use of an unencapsulated pneumococcal culture Avery R36A strain, subline R6 (34, 81-83) which is still being used by most scientists studying *S. pneumoniae* to date.

It was first thought that the ability of *S. pneumoniae* to react with DNA was a result of a mutation or a terminal change undergone by certain abnormal cells (34). It was demonstrated by Hotchkiss in 1954 that this ability to react to DNA is a physiological property. He demonstrated this by growing a culture of R36A strain and periodically taking samples to expose to DNA for 5 min from a single high-step mutant strain resistant to streptomycin. Exposure to DNA after 5 min was terminated by addition of DNase. The number of transformants produced were scored by growing in medium containing streptomycin. Results indicated that after about 3 1/2- 4 hrs of growth, a fraction of the population respond to the DNA and become transformed to resistant cells.

Later Tomasz and Hotchkiss in 1964, demonstrated that the regulation of transformability of pneumococcal cultures were by a macromolecular cell products. They grew two sets of cell. One culture was taken at a competent state (the activator culture) and the other culture was an incompetent culture (detector culture). They found that inoculation of the activator (competent) culture into the detector (incompetent culture) would transfer competence from the activator to the detector cultures. The cultures were

tested for competence by the method described above. To further study this, Tomasz in 1965 set up a similar set of experiments only this time the two cultures were introduced into the separate compartments of a U-shaped glass tube which was divided by a Millipore membrane. This prevented physical contact between the competent and incompetent bacteria. Results indicated that competence was still transferred and that this cell-to-cell transfer of competence did not necessarily require physical contact of the organisms. The activator substance was afterwards isolated from the competent pneumococci (81). It was found that purified preparations of this heat-labile material could convert incompetent bacteria to competence in a fast reaction, the rate depending on the concentration of both the activator and the cell (81). This activator material is what is referred to now as the competence stimulating peptide (CSP) and can be synthetically generated.

Even though the activator material responsible for *S. pneumoniae* competence had been discovered, natural competence was not easily demonstrated nor easily reproducible. There seemed to be several factors affecting development of competence. Some of which were environmental and some cellular. These factors also appear to vary from strain to strain.

Common Factors Affecting *S. pneumoniae* Natural Transformation

i.) Cell density. Tomasz in 1965 demonstrated that the time needed to reach a maximal frequency of competence in a growing culture was found to be a roughly inverse function of the cell concentration (81). It was found that the delay in the expression of competence could be considerably shortened if dilute cultures of pneumococcal were

concentrated to a higher cell density. Different inocula sizes ($10^4 – 10^6$) were used and at frequent intervals, samples were taken and tested for competence using the methods described above. To date competence in *S. pneumoniae* has been demonstrated at densities ranging from $10^6 – 10^8$ (23, 64). It has been suggested that in the natural environment (the nasopharynx) this could be important because if the cell density critical for competence is close to that which triggers host defenses, competence could then serve for adaptation of *S. pneumoniae* to host defense-generated stress (15).

ii.) pH. In *S. pneumoniae*, an increase of the pH of the medium continuously moved the competence peak to earlier exponential phase and thus to lower cell densities (48). A slightly basic pH seems to be optimal (7.4 to 8.0) and no competence is detected at pH less than 7.0 (23, 48, 49, 81, 83). The reason being that the CSP is more active at alkaline than neutral pH (48).

iii.) Calcium. In *S. pneumoniae* Ca^{2+} is essential for growth, competence development, and autolysis (48, 49). Competence is induced optimally at 1 mM Ca^{2+}. Genetic evidence for a Ca^{2+} transporter, mediating the Ca^{2+}- dependent development of competence, came from studies with a mutant which was nontransformable (and resistant to autolysis) in high- Ca^{2+} medium and which was resistant to a specific inhibitor of calcium transporter, 2', 4' –dimethylbenzamil (48). It has been suggested that induction of competence is a response to stress produced by high concentrations of Ca^{2+} (1 mM), which may prevail in the natural environment of *S. pneumoniae*, the body fluids.

iv.) Albumin (either as purified fraction BSA or as heat-inactivated serum). It was established by Hotchkiss and Tomasz in their pioneering work that serum albumin was required for *S. pneumoniae* natural transformation (34, 81). To date, the addition of

serum has been maintained in transformation experiments (4, 29, 57-59). It has also been stated in a review article on cloning and expression of pneumococcal genes that "serum albumin must be added to all transformation media" (40). It has been suggested that albumin may function to stabilize or limit the absorption of certain competence factors to *in vitro* surfaces (49).

v.) Oxygen. Oxygen availability is a major determinant for competence development in exponentially growing cultures of *S. pneumoniae* (14). It is known that NADH oxidase activity is required for optimal competence in cultures grown aerobically but the exact role of oxidative metabolism in competence regulation is still under investigation (14).

vi.) Capsule. Although transformation has long been described as a characteristic of the pneumococci, most studies involving this process have focused on a few laboratory strains, mainly descended from a single unencapsulated subclone (R36A), for which optimal media and protocols were developed (8, 66). All successful natural transformation experiments (that is, without addition of exogenous CSP) to date have been conducted with unencapsulated strains (8, 48, 52, 66). In contrast, strains of clinical importance are encapsulated since the capsule is a major virulence factor. The presence of capsule abolishes competence for natural genetic transformation (8, 48, 66). Several surveys conducted with a collection of clinical isolates of virulent, encapsulated *S. pneumoniae* showed them to all be nontransformable (48, 66). However, some of these isolates developed competence when induced by exogenously added competence factor of a highly transformable laboratory strains.

In depth studies were done on 42 encapsulated strains by one group of scientists using the competence stimulating peptides derived from two different unencapsulated strains (CSP-1 and CSP-2). These CSPs have been previously studied and shown to differ in their *comC* genes (66). These two genes are widely distributed in *S. pneumoniae.* First, the nucleotide sequence of *comC* in each strain was determined for both strands. The allele *comC2* was found in 12 strains, while *comC1* was present in the other strains. The synthetic peptides (CSP-1 and CSP-2) were used to induce competence in the 42 strains. 22 of the 42 (48%) became competent after addition of the CSPs, but none of the strains were competent in parallel cultures without added peptide. Two of the strains responded to both CSP-1 and CSP-2, while each of the others that became competent responded only to the pheromone encoded by its own *comC* allele.

Several reasons have been suggested for nontransformability of natural isolates: (i) the presence of the capsule, which may constitute a physical barrier for the excretion and penetration of the produced competence factor; (ii) the presence of a defect in the production or excretion of the competence factor in some strains; and (iii) a defect in the DNA-processing machinery in strains which were nontransformable even with exogenously added competence factor (48).

Genetic Manipulations of *S. pneumoniae*

The information provided by genomic sequencing has heightened expectations of solutions to biological networks. This cannot however be solved by genomic analysis alone. It is necessary to combine genomic analysis with traditional genetic manipulation. The genetic manipulation of *S. pneumoniae* is made easier because it is a natural

transformant. Studies have shown that by just mixing cloned *pbp1a* and *pbp2x* genes from resistant strains with naturally competent susceptible strains can transform the susceptible strains to the full level of the antibiotic resistance (16).

Several genetic transfer vectors have been described for use in *S. pneumoniae* such as the *E.coli – Streptococcus* shuttle vector, pDP28 and pJDC9 (3, 74, 88). An example of a specific genetic system that has been used to generate a better understanding of resistant mechanisms by Weber et al, 2000. The study investigated the function of the *fib* locus (factor important for bet-lactam resistance). The *fib* locus was discovered by using homology sequences to femAB operon of *S. aureus*. In *S. aureus*, several genetic loci called *fem* are essential for the expression of methicillin resistance, and insertional inactivation of *femA* and *femB* completely abolishes ß-lactam resistance. Weber et al, found two genes with homology to *S. aureus femA/B* in the *S. pneumoniae* genome sequence, named *fibA* and *fibB*. In order to investigate the function of the *fib* locus, loss-of-function mutants were constructed by insertion-duplication mutagenesis, using the plasmid pJDC9. The plasmid cannot replicate in *S. pneumoniae*, but contains an erythromycin resistance gene selectable in this organism. An internal gene fragment of *fibA* was cloned into pJDC9 and transformed into *S. pneumoniae*. Erythromycin-resistant transformants were readily obtained; integration of the plasmid into the chromosome by homologous recombination via the inserted *fibA* gene fragment, and hence disruption of *fibA*, was verified by PCR analysis of the transformants. In all ß-lactam-resistant transformants, disruption of the same *fib* locus resulted in an extreme reduction of the resistance phenotype.

One of the most important developments in *S. pneumoniae* genetic manipulations came recently with the development of intergrational plasmids for the tetracycline-regulated expression of genes in *S. pneumoniae* (78). This was based on gene knock-out technologies which are commonly used to determine the essentiality of a given protein for bacterial growth and survival. If an essential gene is disrupted, no viable bacterial cells are obtained. This is only negative evidence. In order to obtain positive indications for the essentiality of a given gene, Stieger et al developed this controllable knock-out system. Plasmids were designed that allow fusion of a tetracycline controllable promoter to any gene of choice on the streptococcal chromosome (78). The usefulness of the system was demonstrated by the tetracycline-regulated production of firefly luciferase in *S. pneumoniae*, which can be induced fivefold by the addition of tetracycline. By using two promoters of different strength and depending on the presence or absence of tetracycline, an 80-fold range of luciferase activities can be covered. The system was also used to construct strains that depend on the addition of tetracycline for the production of the A subunit of DNA gyrase, an essential streptococcal protein. The growth of such a strain depends on the addition of tetracycline to the medium. In the absence of tetracycline, the cells cease to grow and are not viable. The system presented by Stieger et al, should be useful for the characterization of gene networks in *S. pneumoniae*.

S. pneumoniae Genome

To date, there are three *S. pneumoniae* genomes that have been sequenced completely and are publicly available. The genomes are from different strains namely; a 19F strain sequenced by Glaxo-SmithKline, a type 4 strain sequenced by TIGR and strain

R6 by Eli Lilly (13). Strain R6 is a descendant of the type 2 capsule clinical isolate used by Avery and coworkers to demonstrate the genetic function of DNA, and it is used worldwide as a standard laboratory strain.

The R6 strain was reported to be 2,038,615 bp and 40% G + C content. It contains 2, 043 predicted protein coding regions and 73 noncoding RNA genes (32). As a consequence of the capacity of *S. pneumoniae* to take up DNA, its genome is littered with genes that are apparently derived from other bacteria. There are 40 ORFs that are similar to genes in gram-negative bacteria and that have not been found in other gram-positive genome sequences (32). This is not surprising, because *S. pneumoniae* occupies the same niche in the human respiratory system as several gram-negative species such as *Haemophilus influenzae*. Additionally, at least 2% of the genes were found to be significantly truncated relative to orthologous genes characterized in other bacteria. Transporters are the most frequently truncated genes and it was shown that there are five ORFs that are similar to genes encoding drug efflux pumps (32).

The virulent isolate sequenced by TIGR was shown to have 2,160,837 base pairs with G + C content of 39.7%. It contains 2236 predicated coding regions; of these, 1140 (64%) were assigned a biological role (79).

Glaxo-SmithKline presented sequences and functional annotations for the 2.1 Mbp of a 19F strain which was shown to have 2,046 open reading frames (19).

Table 2: Summary of the Three Available *S. pneumoniae* Genome Data

Strain	Sequencing Company	Genome Size (bp)	ORFs
Strain R6	Eli Lilly	2,038,615	2,043
Type 4 strain	TIGR	2,160,837	2,236
Type 19F strain	Glaxo-SmithKline	~2,100,000	2,046

CHAPTER II

RESEARCH GOALS

The emerging levels of antibiotic resistance exhibited by *S. pneumoniae* strains has become a serious concern for the medical community hence the establishment of the Pneumococcal Molecular Epidemiology Network (PMEN). This network was established in 1997 under the auspices of the International union of Microbiological Societies with the aim of standardizing the nomenclature and classification of resistant pneumococcal clones worldwide. Specific objectives of the network include the following; international collaborations and training support between laboratories and institutions; increased knowledge of resistance and spread of pneumococcal worldwide; standardization of nomenclature of national and international clones; identification of clones using pulse-field gel electrophoresis (PFGE), BOX-PCR, and multilocus sequence typing (MLST); availability of reference isolates of each clone from the American Type Culture (ATCC) Collection, and access to information through a website (53).

The first phase of my research focused on two hypotheses:

1. We hypothesize that resistant strains of *S. pneumoniae* exhibit altered penicillin-binding proteins (PBPs) that differ from PBPs in susceptible strains, as a mechanism of resistance to β-lactam antibiotics.

To test this hypothesis, we propose to determine susceptibility patterns of all clinical isolates to different β-lactam antibiotics. We also propose to study membrane fractions of any highly resistant strains to determine if any proteins correlate with resistant associated PBPs based on their molecular masses and peptide sequences.

2. We hypothesize that *S. pneumoniae* strains may express different protein molecules in their cell envelope that function as virulence factors and/or

epidemiological markers that could be used to distinguish between different clones.

To test this hypothesis, we propose to use SDS-PAGE to generate whole cell protein profiles of all our strains to determine similarities and differences in protein patterns among strains.

Data generated from the initial characterization of 17 *S. pneumoniae* clinical isolates, showed that one strain (Sp D2) exhibited high level resistance to β-lactam antibiotics and also expressed a unique membrane-bound protein in 1D-SDS-PAGE gels. Then the membrane fraction was isolated and further analyzed by MALDI-MS analysis and Edman degradation sequencing. The resulting peptide mass fingerprints and amino acid sequences did not produce any significant matches when searched against all available sequence data, which included the published *S. pneumoniae* genomes. This led to collaborations with Dr. Richard Facklam of the Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, CDC, to further characterize Sp D2 and determine if it exhibits criteria established for typical *S. pneumoniae*.

3. We hypothesize that Sp D2 may express genes that encode for a pneumococcal capsular serotype that is atypical, and that this strain may contain genes that encode for other virulence factors, or that this strain may be incorrectly classified.

To test these hypotheses, we propose to further characterize all strains by serotyping using the antipneumococcal capsular antibodies and use PCR assays to analyze all strains for the presence of the following genes: the *PsaA* that encodes for the pneumococcal surface antigen A, the *LytA* that encodes autolysin, and the *Ply* that encodes pneumolysin.

CHAPTER III

MATERIALS AND METHODS

Growth Conditions and Storage of Test Organisms

All organisms used in this study are listed in Table 4. Stock cultures of all strains were maintained in Mueller Hinton broth plus 15% glycerol and stored in a Revco freezer at -80^0C and sub-cultures of working stock were stored for short periods of time at -20^0C. Organisms were grown on Mueller-Hinton agar (Difco) supplemented with 5% defibrinated sheep blood (Remel). For broth cultures, the strains were grown on liquid media containing BHI (Difco) supplemented with 0.2 % yeast extract (Difco) and 5% casein (Difco) Cultures were incubated at 37^0C and 5% CO_2 in a water-jacketed incubator or in a candle jar. Prior to each experiment, purity of the strains were determined by streaking on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood and observed for the characteristic alpha-hemolysis. In addition, Gram stains and bile solubility tests were performed for quality control.

Table 3: *Streptococcus pneumoniae* Test Strains

Organism	Strain	Source
Sp 83	Vaccine strain	CDC, Atlanta, GA
Sp 86	Vaccine strain	CDC, Atlanta, GA
Sp 95	Vaccine strain	CDC, Atlanta, GA
Sp 105	Vaccine strain	CDC, Atlanta, GA
Sp 117	Vaccine strain	CDC, Atlanta, GA
Sp 125	Vaccine strain	CDC, Atlanta, GA
Sp D1	Clinical isolate	Duke University, NC
Sp D2	Clinical isolate	Duke University, NC
Sp D3	Clinical isolate	Duke University, NC
Sp D4	Clinical isolate	Duke University, NC
Sp D5	Clinical isolate	Duke University, NC
Sp D6	Clinical isolate	Duke University, NC
Sp D7	Clinical isolate	Duke University, NC
Sp D8	Clinical isolate	Duke University, NC
Sp D9	Clinical isolate	Duke University, NC
Sp D10	Clinical isolate	Duke University, NC
Sp D11	Clinical isolate	Duke University, NC
Sp D12	Clinical isolate	Duke University, NC
Sp G-25053	Clinical isolate	Grady Hospital, Atlanta, GA
Sp G-31159	Clinical isolate	Grady Hospital, Atlanta, GA
Sp G-5481	Clinical isolate	Grady Hospital, Atlanta, GA
Sp G-31483a	Clinical isolate	Grady Hospital, Atlanta, GA
Sp Original	Clinical isolate	Boston Hospital, Boston, MA

Kirby-Bauer Susceptibility Tests

All strains were tested for susceptibility to β-lactams (ampicillin, ceftazidime and amoxicillin) and a glycopeptide (vancomycin) using the Kirby-Bauer test (Dispens-O-Disc). The antibiotics were obtained from a commercial source (Difco; Becton Dickinson). *S. pneumoniae* cultures were grown on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood overnight. Several colonies were taken from these plates using sterile cotton swabs, suspended in sterile BHI supplemented with yeast extract and casein. The turbidity was adjusted comparable to a 0.5 McFarland standard using a spectrophotometer 20. A sterile cotton swab was used to inoculate the entire surface of several blood agar plates with the standardized culture to produce a confluent lawn of growth on the plate. Antibiotic disks were placed aseptically on the plates. Plates were incubated overnight in an atmosphere of 5% CO_2. Diameter of zones of growth inhibition were measured in millimeters and compared to breakpoint numbers in the manufacturer's interpretive standards. Strains were categorized as susceptible, intermediate, or resistant to each antibiotic tested.

Minimum Inhibitory Concentration (MIC) Test

The MIC procedure for testing susceptibilities to penicillin G, ampicillin and amoxicillin was performed according to the NCCLS (National Committee for Clinical Laboratory Standards).

i.) Preparation of Organisms. Each *S. pneumoniae* strain was grown on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood overnight. Several colonies were taken from these plates using sterile cotton swabs, suspended in sterile BHI

supplemented with yeast extract and casein. Each culture suspension was adjusted according to a 0.5 McFarland standard using a spectrophotometer 20. The suspensions were further diluted 1:100 in the same media.

ii.) Preparation of Antibiotic Solution. A stock solution of antibiotic was made by suspending 0.1g of antibiotic in 20 ml sterile distilled water resulting in 5mg/ml concentration. 2 ml of the stock was added to 98 ml of sterile BHI supplemented with yeast extract and casein. This corresponds to100 µg/ml antibiotic.

iii.) MIC Dilution Scheme. A series of 15 sterile 13 x 100 mm test tubes per antibiotic for each organisms to be tested was set up. Each tube contained 2 ml of sterile broth. Tube 1 and the negative control contained 50 µg/ml of antibiotic. Two-fold serial dilutions of antibiotic were prepared in the remaining tubes. 2 ml of the *S. pneumoniae* suspension was added to all tubes except the negative control. This halved the antibiotic concentrations in all tubes except the negative control. Tubes were incubated for 12 hrs at 37^0C and 5% CO_2.

iv.) Results interpretations. The MIC was determined by observing all tubes for visible growth. The MIC was defined as the lowest concentration of antibiotic at which the organism did not grow. Strains were categorized as susceptible, intermediate, or resistant to each antibiotic tested based on the standards set for *S. pneumoniae* (NCCLS).

Bile Solubility Test

Strains were streaked onto Mueller-Hinton agar supplemented with 5% defibrinated sheep blood from frozen stocks. Cultures were incubated at 37^0C and 5% CO_2 overnight (approximately 16 hrs). A cell suspension of the strains was made in BHI

broth supplemented with yeast extract and casein. The suspension was then standardized to approximately 40% T using a spectrophotometer. 400 µl of deoxycholate was added to 4 ml of each suspension. The transmittance was then monitored using the spectrophotometer 20. Significant increase in transmittance was considered a positive test for bile solubility. A strain of *Moraxella catarrhalis* was used as a negative control.

Optochin Test

The optochin test is a presumptive test that is used to identify strains of *Streptococcus pneumoniae*. Optochin is a surface active agent that specifically interacts with the cell membrane of *S. pneumoniae* to produce cell lysis. Optochin (ethyl hydrocupreine) disks are placed on inoculated blood agar plates. Because *S. pneumoniae* is not optochin resistant, a zone of inhibition will develop around the disk where the bacteria have been lysed. This zone is typically 14mm from the disk or greater. This test is usually performed on strains that may appear atypical or are not able to be serotyped

Assay for β-lactamase Production by Cefinase Disk Test

The Cefinase disk test is a qualitative method to determine if organisms produce β-lactamases. The Cefinase disks were obtained from a commercial source (Difco, Bacton Dickinson). If the organisms produces a β-lactamase enzyme, the disk turns red as the chromogenic cephalosporin, nitrocefin is cleaved. If no color change occurs, then the organism does not produce a β-lactamase enzyme. A sterile Cefinase disk moistened with a drop of sterile water was placed into a sterile petri dish. From a *S. pneumoniae* culture grown overnight on a Mueller-Hinton blood agar plate, several well-isolated colonies

were removed using a sterile wire loop and smeared on the Cefinase disk. The disk was observed for a color change from its original yellow color to red. *Moraxella catarrhalis* was used as a positive control. A positive reaction suggests penicillin resistance by organisms that produce β-lactamases.

Assay for Degradation of β-lactams by Sp D-2

An assay was used to determine whether the clinical isolate Sp-D2 that showed the highest level of resistance was in some way degrading the β-lactams as a mechanism of resistance. Sp-D2 was grown in the presence of 8 μg/ml of amoxicillin. (Sp-D2 MIC to amoxicillin is 31.25 μg/ml). After incubation overnight under standard conditions, the media was filtered to remove Sp-D2 cells. Serial dilutions of the filtered media were prepared with fresh media. Each tube was then inoculated with Sp Original whose MIC to amoxicillin is 0.12 μg/ml. If the antibiotic was degraded by Sp-D2, then Sp Original would be expected to grow in the dilution tubes that initially had high amoxicillin concentrations. As a positive control we used a strain of *Moraxella catarrhalis* obtained from Duke University that has been shown to produce β-Lactamases.

Serotyping

The Quelling reaction has been the "gold standard" method for typing of *S. pneumoniae* . The conventional method of serotyping *S. pneumoniae* uses pneumococcal typing antisera. The quellung reaction (swelling reaction) forms the basis

of serotyping and relies on the swelling of the capsule upon binding of anticapsular antibody (44) .

SDS-PAGE of Whole-Cell Proteins of *S. pneumoniae* Strains

Strains were analyzed using SDS-PAGE of whole-cell proteins.

i.) Preparation of Whole-Cell Lysates. Each frozen strain was streaked on to Mueller-Hinton agar supplemented with 5% defibrinated sheep blood, then isolated colonies were Gram-stained to examine purity. Cells from plates were suspended in 50 mM Tris buffer and adjusted to an optical density of 0.4 at 600 nM using a spectrophotometer 20. One ml of each culture suspension was transferred to a microcentrifuge tube and centrifuged for 1 minute at 4,400 x g. The supernatant was discarded via aspiration. The pellets were then resuspended in 30 ul of sample buffer and boiled for 10 minutes at 100^0 C water bath.

ii.) Preparation of Gels. Polyacrylamide gels were prepared as described by Laemmli (41). The discontinuous vertical system composed of an upper stacking gel containing 6% polyacrylamide and a lower running gel containing 12.5 % polyacrylamide. The components of the running gel were first prepared and degassed for 15 minutes. Then, TEMED was added, mixed and immediately poured into the vertical apparatus. The gel was allowed to polymerize for approximately one hour. The components of the stacking gel were then prepared, degassed for 15 minutes and after addition of TEMED, was poured slowly over the running gel. The comb was inserted and allowed to polymerize for 30 minutes.

iiii.) Electrophoresis. 10 µl of each sample was loaded into the designated wells. 10 µl of the molecular weight standard (2 µl standard in 18 µl sample buffer) was also loaded into each gel. The gel was electrophoresed at 100 V for 30 min until the dye front reached the top of the running gel. The voltage was then reduced to 70 V and run for approximately 7 hours or until the dye front reached about 1 inch from the bottom of the gel. The gel was then stained in Coomassie blue for 15 minutes with shaking. The gel was then rinsed in distilled water and destained for 30 minutes. The gel was then left to further destain overnight in a pan of distilled water with a few drops of chloroform before photographing it.

S. pneumoniae Cytoplasmic Membrane Isolation

Penicillin-binding proteins are located in the cytoplasmic membrane hence requiring a membrane extraction. *S. pneumoniae* strains were grown in 1 liter of BHI supplemented with yeast extract and casein up to the mid-logarithmic growth phase (approximately 7 hrs). Cells were harvested by centrifugation at 4,400 x g for 10 minutes. Pellets were then washed once in 50 mM potassium phosphate and 2 mM $MgCL_2$ buffer and centrifuged for 20 min at 10,000 x g. Resulting cell pellets were resuspended in approximately 30 ml of the same buffer. 300 µl of 100 mM PMSF (phenylmethylsulfonyl fluoride) was added and mixed. 50 µg/ml DNase I (Amoroso et el, 2001) was added and then the cells were passed through a French press 3 times at 1,000 kg/cm^2. Unbroken cells were removed by centrifugation at 10,000 x g for 20 minutes twice. Another 300 µl of PMSF was added to the supernatant. The supernatants were then ultracentrifuged at 110,000 x g for 2 hrs and 10 min at 15^0C (1, 95). The resulting membrane pellets were

solubilized in TritonX and stored at -20^0C. The protein concentration of the membrane fraction was determined using the Bradford method.

Detection of Penicillin-Binding Proteins with Bocillin

The procedure used for detection of *S. pneumoniae* penicillin-binding proteins was described by Zhao et al. BOCILLIN FL, a fluorescent penicillin was obtained commercially (Molecular Probes). The 1 mg content of BOCILLIN was dissolved in 1 ml of Methanol. Various concentrations of BOCILLIN were prepared by diluting in sterile distilled water. The *S. pneumoniae* frozen membrane fractions were thawed and various concentrations of these were prepared. 75 µl of each protein concentration was mixed with 25 µ of BOCILLIN and incubated in a 35^0C water bath for 30 minutes. The reaction mixture was then denatured with 100 µl SDS-denaturing solution at 100^0C for 3 minutes. 10 µl of each reaction was then subjected to SDS-PAGE. The polyacrylamide gel was prepared and run as described in the SDS-PAGE of whole cell section (see page 32). After running, the gel was rinsed off in distilled water and visualized under UV light (95).

Generation of *S. pneumoniae* Step Mutants

Gradient plates were prepared by pouring 20 ml of BHI supplemented with yeast extract and casein onto petri plates and letting them solidify at an angle to create a gradient to a point of zero. The plates were then leveled and 20 ml of BHI supplemented with yeast extract and casein containing 2 ug/ml ampicillin (this concentration was the

initial concentration used but was increased with time) were poured into each plate and allowed to solidify.

Strains were removed from the freezer and streaked onto Mueller-Hinton agar supplemented with 5% defibrinated sheep blood. The plates were incubated at 37^0C in a candle jar overnight (15-16 hrs). From each plate, isolated colonies were used to make a suspension in sterile saline. 0.2 ml of each strain was spread onto the 2ug/ml gradient plates using the spread plate method. The plates were then inverted and grown under the same conditions for 24 hrs. Isolated colonies growing at the highest concentration of ampicillin were streaked on the same plate towards the higher concentration areas of the plates. The plates were re-incubated under the same conditions. The colonies were then picked and transferred to another fresh plate containing 2 ug/ml ampicillin. The above procedure was then repeated with plates containing higher concentrations of ampicillin. Strains were frozen periodically when they attained certain levels of ampicillin (2 ug/ml, 3 ug/ml, 5 ug/ml, 7 ug/ml). The concentrations at which the mutants grew were verified by plating on fresh media containing various ampicillin concentrations.

DNA Isolation of *S. pneumoniae* Strains

The method used for isolation of *S. pneumoniae* DNA is a modified procedure by Whatmore et al. *S. pneumoniae* strains were grown in 500 ml of BHI supplemented with yeast extract and casein at 37^0C and 5% CO_2 overnight (approximately 16 hrs). Cells were harvested by centrifugation at 4,400 x g for 10 minutes. Cell pellets were resuspended in 5 ml of a solution containing 50 mM Tris-HCL and 10 mM EDTA adjusted to pH 8.0. 25 µl of 20 mg/ml lysozyme was added and the suspension was

incubated in a 37^0C water bath for 10 min. 25 µl of 10 mg/ml proteinase K was added and incubated for another 30 min. After incubation, 200 µl of 20% Sarkosyl was added and mixed. The final suspension was then subjected to a phenol extraction and a chloroform extraction. The DNA was then precipitated in 95% cold ethanol with 10% 3 M sodium acetate (pH 5.2). DNA was spooled using a glass rod and placed in clean vials (89). The DNA was resuspended in 1-2 ml of sterile distilled water with a few drops of chloroform and stored at room temperature. DNA concentration was determined using a UV Spectrophotometer.

Trypsin In-Gel Digest

In order to study the unique protein band exhibited by strain Sp D2, it was necessary to extract proteins bands from the SDS-PAGE gels. The ProteoProfile™ Trypsin In-Gel Digest kit was used for the purpose (Sigma). Protein bands from 1D SDS-PAGE gels were processed according to the manufacture procedure. The bands of interest were excised using a clean scalpel and placed in siliconized eppendorf tubes, one gel piece per tube. Each gel piece was covered with 200 µl of destaining solution (containing 200 mM ammonium bicarbonate and 40% acetonitrile) and incubated at 37^0 C for 30 minutes. The solution was then discarded and the step repeated one more time. The gel pieces were dried on Kim Wipes. 20 µl of the prepared trypsin solution was then added followed by 50 µl of the trypsin reaction buffer. The gel pieces were incubated for 8 hours at 37^0 C in a sonic bath (Fisher Scientific, Bransonic). After incubation, the liquid from the gel was transferred to new siliconized eppendorf tubes. 50 µl of the peptide extraction solution was added to the gel pieces and re-incubated for 30 minutes. The

peptide extraction solution was then removed and combined with the liquid from the first incubation (5, 93, 94).

Preparation of Peptide Samples for Mass Spectrometry Analysis

Peptide samples were purified and concentrated by use of a Zip Tip (Millipore) which has C_{18} resin fixed at its end and rinsed according to the manufacture's instructions in 10 µl of 0.1% trifluoroacetic acid (TFA) and 50% acetonitrile (ACN). Peptides were eluted in 10 µl 1:1 ACN-0.1 % TFA. A 0.5 µl volume of the concentrated peptide-containing sample was mixed with 0.5 µl of a saturated solution of α-cyano-4-hydroxycinnamic acid. 0.5 µl of each sample was spotted on the mass spectrometer sample plate (84, 93, 94).

Protein Identification by MALDI-TOF MS

Mass spectra were obtained on a matrix assisted laser desorption and ionization / time-of-flight mass spectrometer (MALDI-TOF), TOF/TOF^{TM} 4700 Proteomics analyzer (Applied Biosystems) at two different core labs, Georgia Institute of Technology and Emory Univeristy core labs.

MALDI spectra were calibrated using a peptide mixture provided by the manufacturer (Applied Biosystems). Protein identifications were realized by the method of peptide mass fingerprinting and database searches (24).

Database Searches for Protein Identification

Monoisotopic peptide masses obtained from mass spectra were searched against the SWISS-PROT, NCBInr and MSDB databases using the MASCOT search program. The following parameters were used in the searches: Bacteria (Eubacteria) or *S. pneumoniae*, MS/MS Ion Search, protein mass of 50 kDa, trypsin digest with two missed cleavages, fragment ion mass tolerance of ± 75 ppm and possible oxidation of methionine (84).

N-terminal Sequencing

Sp D2 protein samples were further analyzed by the Emory core facility. Sp D2 protein samples were subjected to SDS-PAGE/blotting onto PVDF membrane and stained with Amido Black yielding a profile. The major band of interest was excised and sequenced using 18 Edman degradation cycles yielding an N-terminal sequence. WU-BLAST was used to search the Swall database with the N-terminal sequence data

Genetic Analysis of Clinical Isolates for Genes that Encode for Virulence Factors

Pneumolysin gene (*ply*)

In order to further characterize our clinical isolates and to positively identify them as *S. pneumoniae* strains, PCR was performed on the strains targeting genes encoding three major virulence factors; pneumolysin, autolysin and pneumococcal surface antigen.

For pneumolysin, two primers and one probe were selected based on published pneumolysin gene sequence (70). A nested PCR approach was taken. The outer primers (5'-ATTTCTGTAACAGCTACCAACGA-3') and (5'-AATTCCCTGTCTTTTCAAAGTC-3'), amplified a 348-bp region of the pneumolysin

gene. The inner primers (5'-CCCACTTCTTCTTGCGGTTGA-3') and (5'-TGAGCCGTTATTTTTTCATACTG-3'), amplified a 208-bp region (10, 18, 69, 70).

Amplification was carried out in an automated thermal cycler (Perkin-Elmer). The 50 μL reaction mixture contained 50 mM KCL, 10 mM Tris-Hcl pH 8.3, 1.5mM $MgCl_2$, 0.01% (vol/vol) gelatin, 5 mM deoxyribonucleotides, 50 pmol primers, 2.5 units of Tag DNA polymerase (Promega), and various amounts of DNA extracted from the strains. Amplification was done using 30 cycles of denaturation at 94^0C for 1 min, followed by annealing of the primers at 55^0C for 1 min and synthesis at 72^0C for 1 min. Nested amplifications were done in the same manner. Negative control was distilled water and positive control was previously tested pneumococcal DNA. A 10μL volume of the completed reaction mixture was run in a 1.5% agarose gel stained in ethidium bromide (22, 38, 39, 47, 56, 69, 70, 80, 86, 87).

Autolysin gene (*lytA)*

A nested PCR approach was undertaken in the same manner as described for the *ply* amplification. The outer primers used were (5'-GGCTACTGGTACGTACATTC-3') and (5'-AATCAAGCCATCTGGCTCTA-3'). The inner primers used were (5'-ATCCAAAAGACAAGTTTGAGA-3') and (5'-CTGGATAAAGGCATTTGATAC-3'). PCR reaction was carried out in the same manner as described above (55).

Pneumococcal surface antigen gene (*psaA)*

Single primers were used to amplify the *psA* gene. The forward primer was (5'-CTTTCTCTGCAATCATTCTTG-3') and the reverse primer was (5'-

GCCTTCTTTACCTTGTTCTGC-5'). The PCR reaction was otherwise carried out in much the same manner as described above (60).

Discrimination of *S. pneumoniae* by Arbitrarily Primed PCR

In clinical laboratories, identification of *S. pneumoniae* from other streptococci is important especially in cases where strains are not able to be serotyped. If a strain cannot be serotyped, an arbitrarily primed PCR with a single primer M13 universal is used as a method to distinguish *S. pneumoniae* from other upper respiratory tract streptococci (54). Arbitrarily primed PCR was performed on Sp D2.

CHAPTER IV

RESULTS

Kirby-Bauer Susceptibility Tests

The Kirby-Bauer method is a qualitative test to determine if bacteria are susceptible or resistant to an antimicrobial agent. Four antimicrobial agents were tested including three β-lactams (ampicillin, ceftazidime and amoxicillin) and a glycopeptide (vancomycin). All clinical isolates from Duke University and Grady hospital were resistant to ampicillin (Table 4). The Duke strains exhibited extremely high resistance with no zones of inhibition. The resistance break point of *S. pneumoniae* to ampicillin using the Kirby-Bauer method, is any value less than 21 mm diameter of inhibition zone. The vaccine strains and Sp Original, a clinical isolate from Boston hospital showed intermediate susceptible to ampicillin. Only one organism, Sp D2 was resistant to ampicillin, amoxicillin and ceftazidime, . All organisms were susceptible to vancomycin.

TABLE 4: Kirby-Bauer Susceptibility Results for *S. pneumoniae*

[a]Strain	Ampicillin 10 mcg	Ceftazidime 30 mcg	Vancomycin 30 mcg	Amoxicillin 30 mcg
Sp D-1	14 (res)	20 (sus)	20 (sus)	27 (sus)
Sp D-2	0 (res)	10 (res)	25 (sus)	12 (res)
Sp D-3	0 (res)	21 (sus)	25 (sus)	30 (sus)
Sp D-4	0 (res)	20 (sus)	22 (sus)	30 (sus)
Sp D-5	0 (res)	16 (int)	22 (sus)	20 (sus)
Sp D-6	0 (res)	21 (sus)	24 (sus)	32 (sus)
Sp G-25053	15 (res)	22 (sus)	24 (sus)	34 (sus)
Sp G-31159	13 (res)	25 (sus)	21 (sus)	31 (sus)
Sp G-5481	12 (res)	22 (sus)	22 (sus)	30 (sus)
Sp G-31483a	14 (res)	21 (sus)	23 (sus)	34 (sus)
Sp Original	29 (int)	34 (sus)	24 (sus)	48 (sus)
Sp 83	24 (int)	32 (sus)	24 (sus)	43 (sus)
Sp 86	26 (int)	32 (sus)	24 (sus)	40 (sus)
Sp 95	25 (int)	31 (sus)	24 (sus)	38 (sus)
Sp 105	25 (int)	32 (sus)	23 (sus)	38 (sus)
Sp 117	28 (int)	34 (sus)	25 (sus)	42 (sus)
Sp 125	25 (int)	32 (sus)	24 (sus)	40 (sus)
[b]Break point of resistance	< 21 mm	< 14 mm	< 9 mm	< 13 mm

[a] Abbreviation for source of strains: Sp D = Duke strains, Sp G = Grady strains
[b]The interpretive standards: Res = resistant, Sus = susceptible, Int = intermediate

Minimal Inhibitory Concentration (MIC) Test

The minimum inhibitory concentration test (MIC) is a quantitative susceptibility test. In these experiments, a broth macro dilution method was used determine the minimal concentration of antibiotics that will inhibit growth of representative strains. Three antibiotics were tested, penicillin G, ampicillin and amoxicillin all of which are routinely used to treat *S. pneumoniae* infections (Severina et al, 1999). According to the NCCLS guidelines, *S. pneumoniae* is considered resistant if its MIC to penicillin G is > 2 μg/ml, to ampicillin is > 4 μg/ml, and to amoxicillin is > 2 μg/ml. Two strains from Duke University, Sp D1 and Sp D2 were found to be resistant: Sp D1 had a MIC of 6.25 μg/ml for ampicillin and SP D2 had a MIC of 4.0 μg/ml for penicillin G, > 25 μg/ml for ampicillin, and 31.25 μg/ml for amoxicillin (Table 6). One Vaccine strain (Sp 117) was resistant to penicillin G with a MIC of 3.125 μg/ml. Sp Original was susceptible to all three β-lactams tested here. The Grady strains showed varied results: Sp G-25053 was resistant to ampicillin, but intermediate for penicillin G; Sp G31159 and Sp G-5481 were susceptible to both β-lactams tested; and Sp G-31483a was resistant to both penicillin G (MIC of 6.25 μg/ml) and ampicillin (MIC of 25 μg/ml).

TABLE 5: Minimal Inhibitory Concentration (MIC) Results for *S. pneumoniae*

[a]Strain	Penicillin G MIC ug/ml	Ampicillin MIC ug/ml	Amoxicillin MIC ug/ml
Sp D-1	6.25 (res)	not done	not done
Sp D-2	4 (res)	> 25 (res)	31.25 (res)
Sp G-25053	1.56 (int)	9.38 (res)	not done
Sp G-31159	0.048 (sus)	0.39 (sus)	not done
Sp G-5481	0.39 (sus)	0.78 (sus)	not done
Sp G-31483a	6.25 (res)	25 (res)	not done
Sp Original	0.015 (sus)	0.195 (sus)	0.12
Sp 83	0.003 (sus)	0.195 (sus)	not done
Sp 86	0.005 (sus)	0.098 (sus)	not done
Sp 95	0.006 (sus)	0.195 (sus)	not done
Sp 105	0.048 (sus)	0.098 (sus)	not done
Sp 117	3.125 (res)	0.098 (sus)	not done
Sp 125	0.098 (sus)	not done	not done
[b]Break point of resistance	> 2 ug/ml	> 4 ug/ml	> 2 ug/ml

[a] Abbreviation for source of strains: Sp D = Duke strains, Sp G = Grady strains
[b]The interpretive standards: Res = resistant, Sus = susceptible, Int = intermediate

Assay for β-Lactamase Production by Cefinase Disk Test

The Cefinase test was performed on all strains. The Cefinase test determines if organisms produce a lactamase enzyme. The Cefinase disk is similar to an antibiotic disk and is impregnated with a chromogenic cephalosporin. This cephalosporin, called nitrofecin, turns red as the amide bond in the β-lactam ring of the nitrocefin is hydrolyzed

by a β-lactamase enzyme. To date, no *S. pneumoniae* strain has been demonstrated to be β-lactamase positive but over 90% of *Moraxella catarrhalis* strains worldwide are β-lactamase positive (9). In this study none of the *S. pneumoniae* strains were β-lactamase positive.

SDS-PAGE of Whole-Cell Proteins of *S. pneumoniae* Strains

A profile of whole-cell proteins from 17 *S. pneumoniae* strains as determined by SDS-PAGE is shown in Figures 3 and 4. Some strains from Figure 3 were repeated in Figure 4 in an effort to obtain better resolved bands. The profile shows some differences and similarities between the test organisms. All of the organisms exhibit several common major bands and a few distinct differences. There are some high-molecular-weight bands that are not exhibited in all of the test strains. For example, test organism Sp D2 exhibited a band at approximately 50 kDa that is not present in any other test strain. The protein band exhibited by Sp D2 at approximately 50 kDa was most unique and highly resolved (Figure 3, Lane 3).

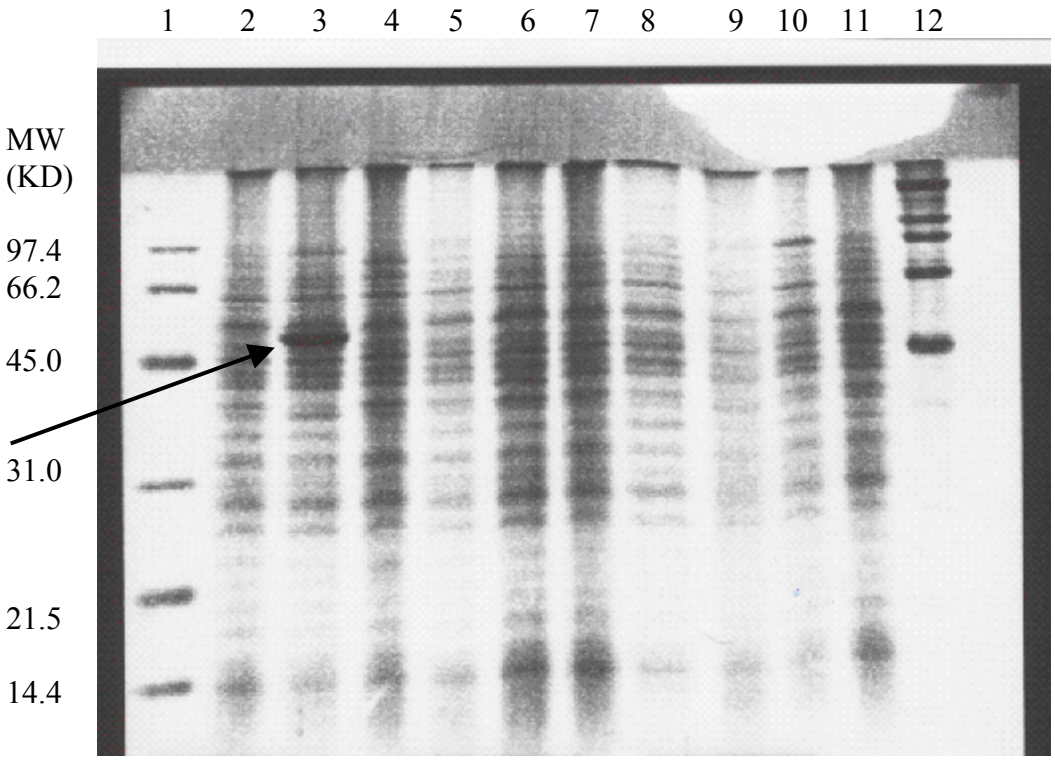

Figure 3: SDS-PAGE of *S. pneumoniae* Whole-Cell Proteins. Lane 1, Low MW marker (97.4, 66.2, 45.0, 31.0, 21.5 kDa); Lane 2, D-1; Lane 3, D-2.; Lane 4, D-3; Lane 5, D-4; Lane 6, D-5; Lane 7, D-6; Lane 8, G-25053; Lane 9, G-31159; Lane 10, G-31483a; Lane 11, G-5481; Lane 12, High MW marker (200, 116.25, 97.4, 66.2, 45 kDa).

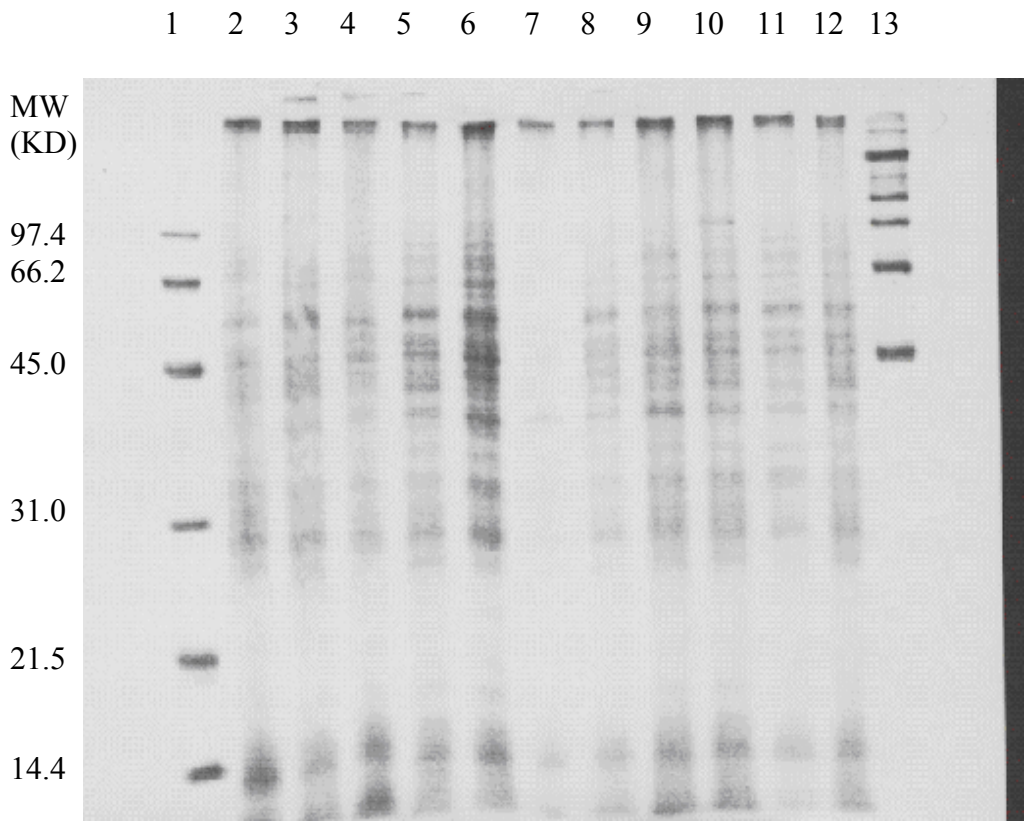

Figure 4: SDS-PAGE of *S. pneumoniae* Whole-Cell Proteins (Continued) .

Lane 1, Low MW marker; Lane 2, Sp Original; Lane 3, Sp 83; Lane 4, Sp 86; Lane 5, Sp 95; Lane 6, Sp 105; Lane 7, empty; Lane 8, Sp 117; Lane 9, Sp 125; Lane 10, G-25053; Lane 11, G-31483a; Lane 12, G-5481; Lane 13, High MW marker (200, 116.25, 97.4, 66.2, 45 kDa).

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Detection of *S. pneumoniae* Penicillin-Binding Proteins (PBPs)

BOCILLIN FL is a commercially available fluorescent penicillin that can be used as a labeling reagent for penicillin-binding proteins (95). In order to determine the optimum range of BOCILLIN FL and membrane protein concentrations required for detection, it was necessary to optimize the system. Serial dilutions of membrane fractions from Sp G-25053 and Sp 95 were made, each of which was labeled with different concentrations of BOCILLIN FL (50 μM and 25 μM . The labeled membrane preparations were separated by SDS-PAGE and detected under UV light (Figure 5). There were two distinct bands (molecular weight not determined). The slower migrating band is faint, and it was later determined that BOCILLIN FL is unstable under UV light thus requiring rapid photography. Results showed that the concentration of BOCILLIN FL used was more significant than the protein concentrations. Fractions labeled with 25 μM BOCILLIN FL (lanes 7 -12) showed less fluorescence than those labeled with 50 μM BOCILLIN FL (all other lanes). There was no obvious difference in fluorescence intensities with the varied protein concentrations.

Due to the rapid loss of fluorescence intensities, this method did not prove to be useful for binding and differential labeling of PBPs in this study.

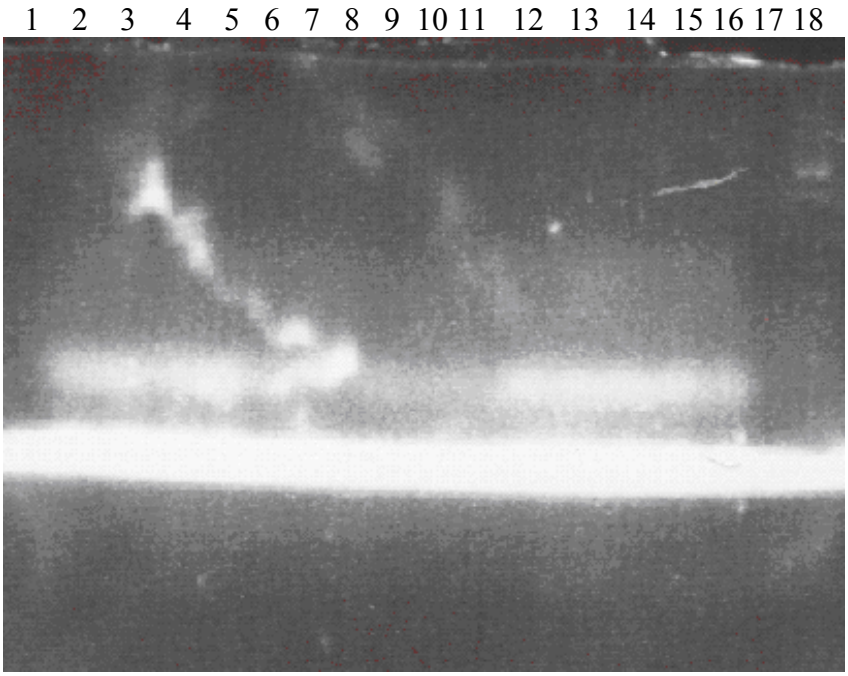

Figure 5: Detection of PBPs of *S. pneumoniae*. Lane 1, Low MW marker (not visible under UV light); Lane 2, 225 µg/ml protein of Sp G25053 and 50 µM Bocillin ; Lane 3, 112.5 µg/ml protein of Sp G25053 and 50 µM Bocillin ; Lane 4, 56.25 µg/ml protein of Sp G25053 and 50 µM Bocillin; Lane 5, 22.12 µg/ml protein of Sp G25053 and 50 µM Bocillin; Lane 6, 14 µg/ml protein of Sp G25053 and 50 µM Bocillin; Lane 7, 225 µg/ml protein of Sp G25053 and 25 µM Bocillin; Lane 8, 112.5 µg/ml protein of Sp G25053 and 25 µM Bocillin; Lane 9, 56.25µg/ml protein of Sp G25053 and 25 µM Bocillin; Lane 10, 28.12µg/ml protein of Sp G25053 and 25 µM Bocillin; Lane 11, 14µg/ml protein of Sp G25053 and 25 µM Bocillin; Lane 12, 27.5 µg/ml protein of Sp 95 and 50 µM Bocillin; Lane 13, 18.75 µg/ml protein of Sp 95 and 50 µM Bocillin; Lane 14, 9.37 µg/ml protein of Sp 95 and 50 µM Bocillin; Lane 15, 4.68 µg/ml protein of Sp 95 and 50 µM Bocillin; Lane 18, high MW marker (not visible under UV light);

Generation of *S. pneumoniae* Step Mutants

Two representative strains Sp G31159 and Sp Original were used to generate step mutants by the gradient plate technique. Ampicillin is routinely used to treat *S. pneumoniae* infections and therefore this β-lactam was chosen to generate the mutants. The first gradient plates contained a maximum ampicillin concentration of 2 μg/ml. Sp Original was stepped up to 5 μg/ml and Sp G31159 was stepped up to approximately 7 μg/ml. These values have been verified by plating on fresh ampicillin plates containing 5 μg/ml and 7 μg/ml respectively. The step mutants were periodically stored in Mueller Hinton broth and glycerol at -20^0 C and -70^0 C while performing these experiments.

Sp D2 Unknown Protein Analysis

Whole cell SDS-PAGE results shown in Figure 3, lane 3, revealed a protein unique to Sp D2 that is approximately 50 kDa. Due to the high level of resistance of this particular strain, this unique protein was of interest. In an attempt to characterize this protein, several procedures were performed. Firstly, Since PBPs are membrane bound enzymes, the membrane proteins of several of the strains were isolated including Sp D2 in attempt to determine if the unique protein is membrane bound. The membrane fractions were run on an SDS-PAGE gel (Figure 6). Results indicate that the unique protein of approximately 50 kDa is present in the Sp D2 membrane fraction (Lanes 2 to 5) shown in Figure 6. Sp Original was used as a control and the unique protein is not present in the Sp Original membrane fraction lanes, and was not present in whole-cell SDS-PAGE of other strains (see Figures 3 and 4).

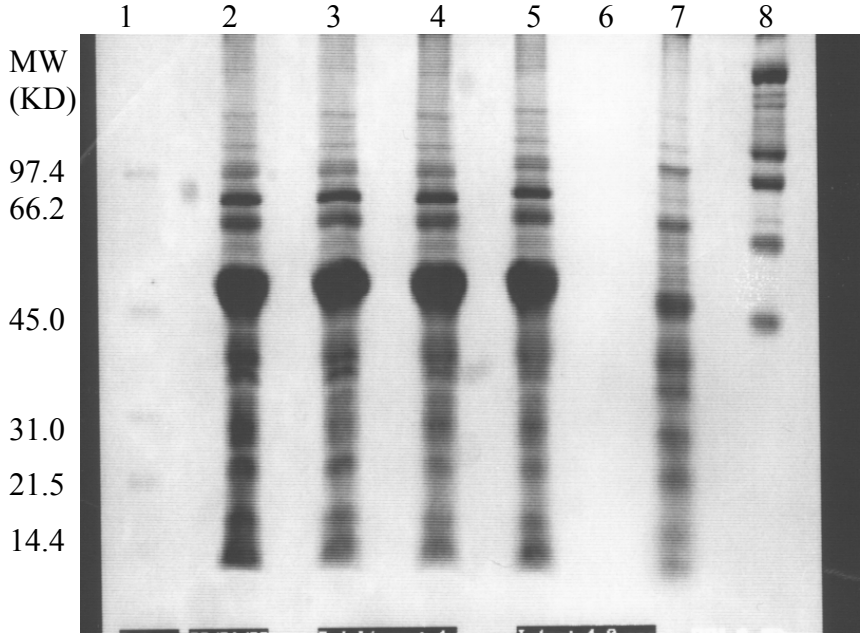

Figure 6: SDS-PAGE of *S. pneumoniae* membrane fractions. Lane 1, Low MW marker; Lanes 2 - 5, Sp D-2; Lane 6, empty, Lane 7, Sp Original; Lane 8 High MW marker (200, 116.25, 97.4, 66.2, 45 kDa). Sp D2 membrane fractions were run on replicated (lanes 2-5) in order to provide more bands to excise for future studies.

Sp D2 Growth Curve with Whole Cell SDS-PAGE Analysis

A growth curve study was also performed on Sp D2 (Figure 7). Cells were sampled periodically and analyzed on SDS-PAGE to determine whether the Sp D2 protein of interest was present throughout the growth period (Figure 8). Based on these results, there was evidence of two protein bands. The bottom band appeared to increase in intensity with time but the top band maintained the same intensity throughout the exponential growth phase.

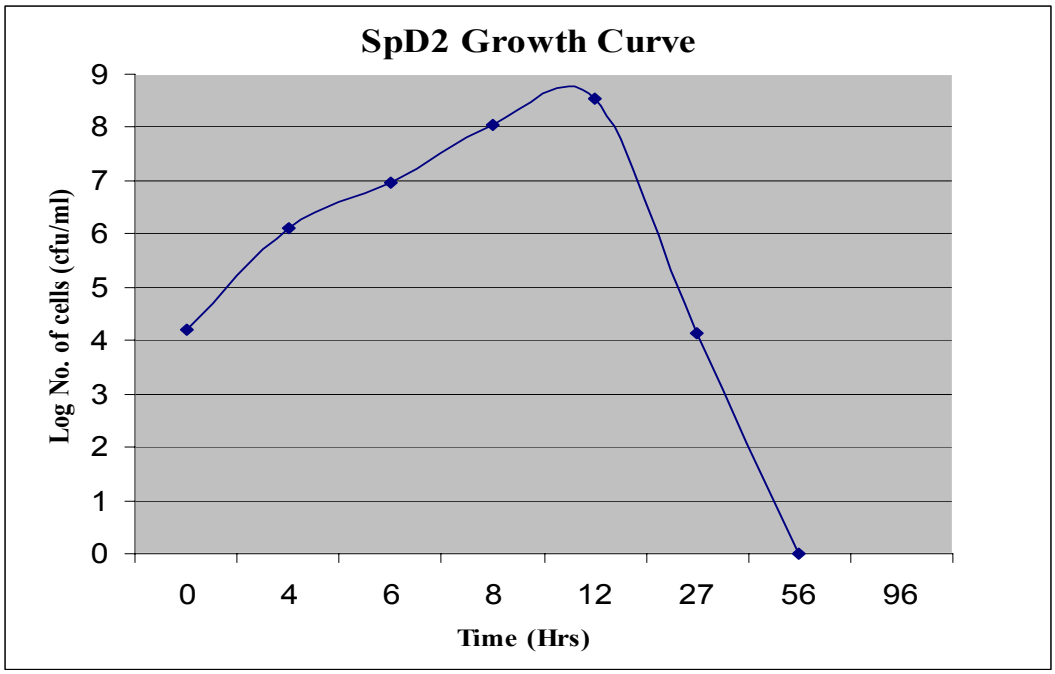

Figure 7: Sp D2 growth curve

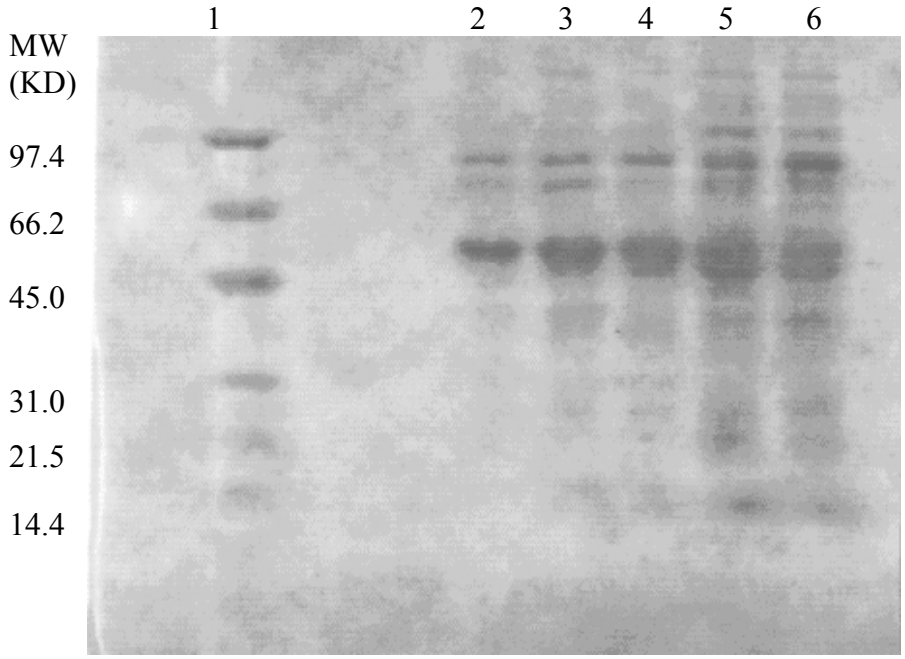

Figure 8: SDS-PAGE of Sp D2 whole cell proteins from growth curve sampling. Lane 1, Low MW marker; lane 2, 6hr growth; Lane 3, 8hr; Lane 4, 12 hr; Lane 5, 28 hr; Lane 6, 50 hr.

MALDI-MS Analysis of Sp D2 Protein Band

The distinct Sp D2 protein band of approximately 50 kDa was excised from the membrane fraction SDS-PAGE gel (See Lanes 2-5, Figure 6) and processed using a trypsin In-Gel digest kit. Trypsin cleaves after Lysine and Arginine residues (except if they are before Proline) therefore producing specific fingerprints for specific proteins.

Resulting peptide digests were then analyzed by MALDI-MS (matrix-assisted laser desorption and ionization mass spectrometry). Figure 9 represents the MALDI-MS data of the digests. The tallest five peaks were selected by an automated procedure and analyzed further by MALDI-MS/MS also referred to as tandem mass spectrometry (Figures 10-14).

Figure 10, represents the 3358 Da peptide, Figure 11, represents the 2894 Da peptide, Figure 12, represents the 2878 Da peptide, Figure 13, represents the 2244 Da peptide, Figure 14, represents the 2102 Da peptide,

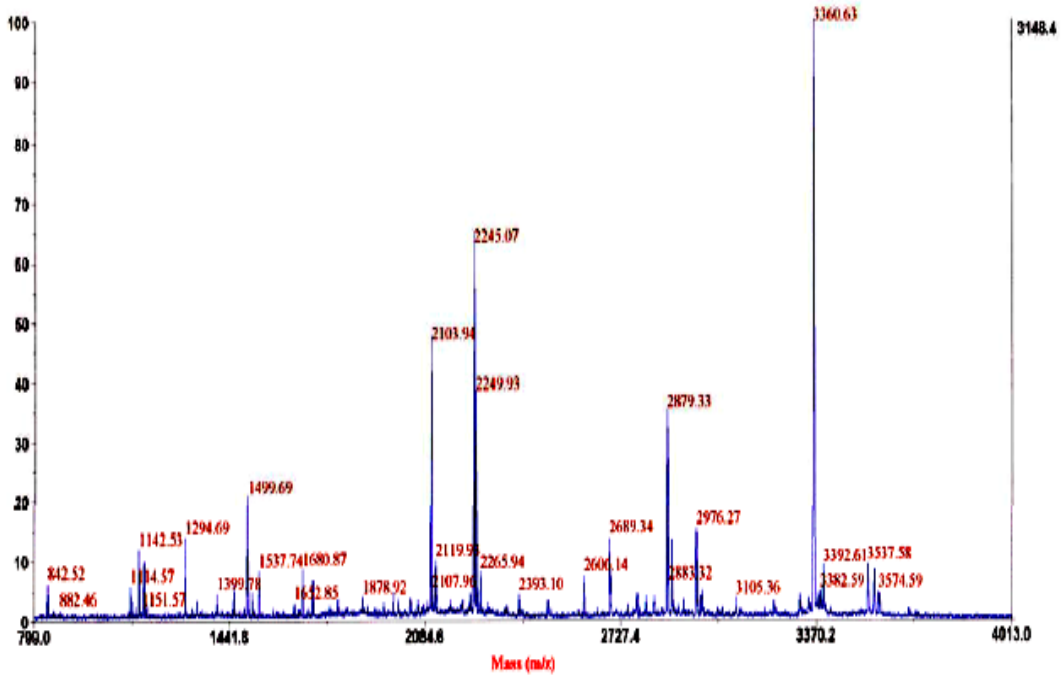

Figure 9: MALDI-MS spectra of Sp D2 trypsin digests.

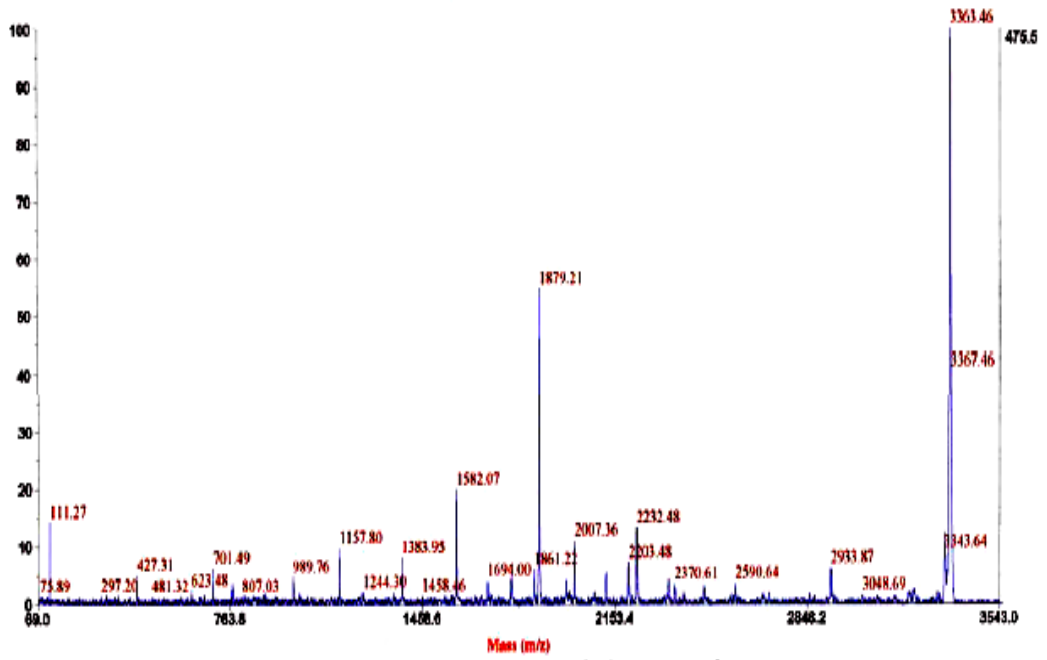

Figure 10: MALDI-MS/MS spectra of Sp D2 3358 Da peptide

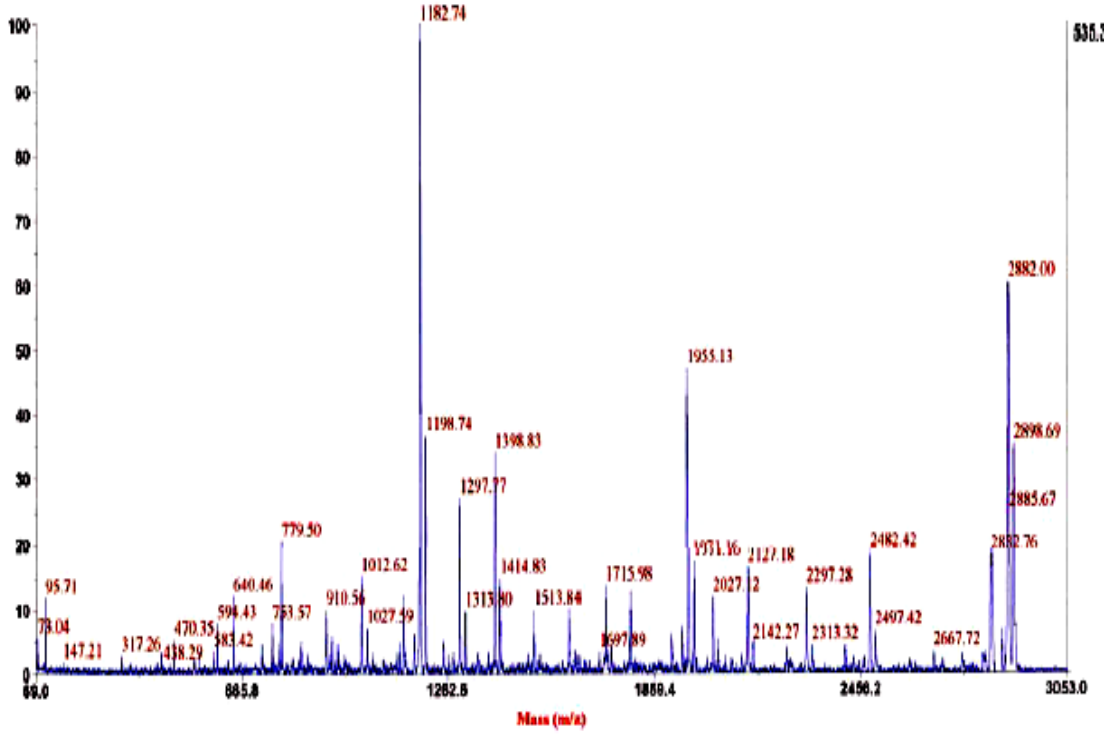

Figure 11: MALDI-MS/MS spectra of Sp D2 2894 Da peptide

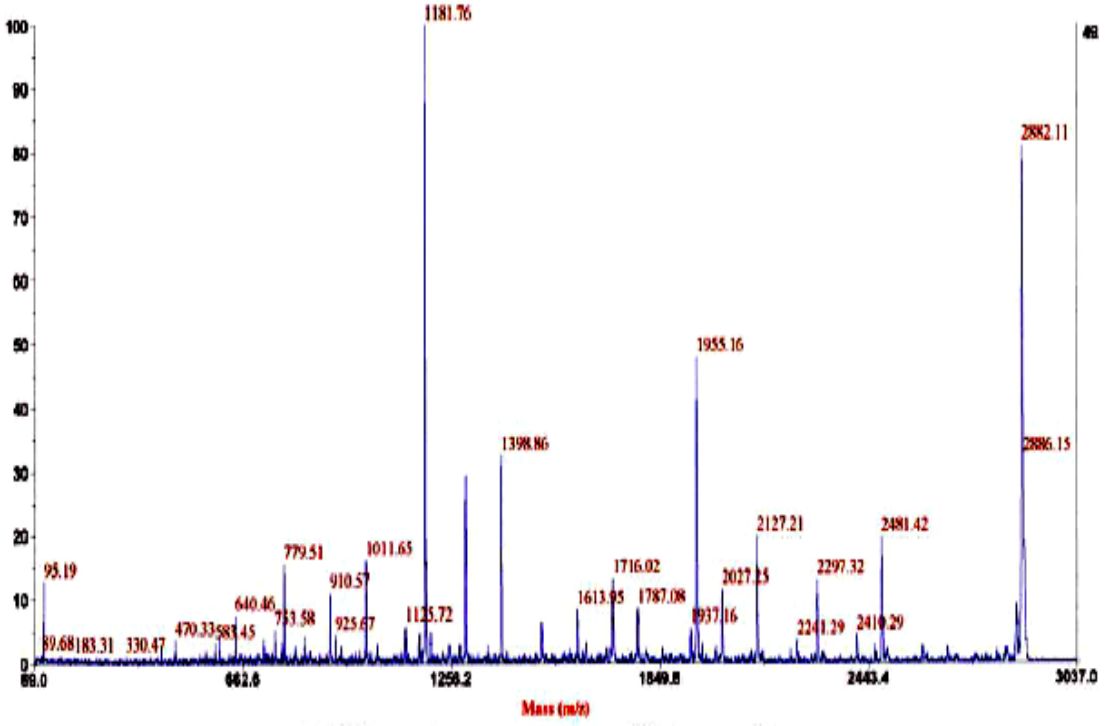

Figure 12: MALDI-MS/MS spectra of Sp D2 2878 Da peptide

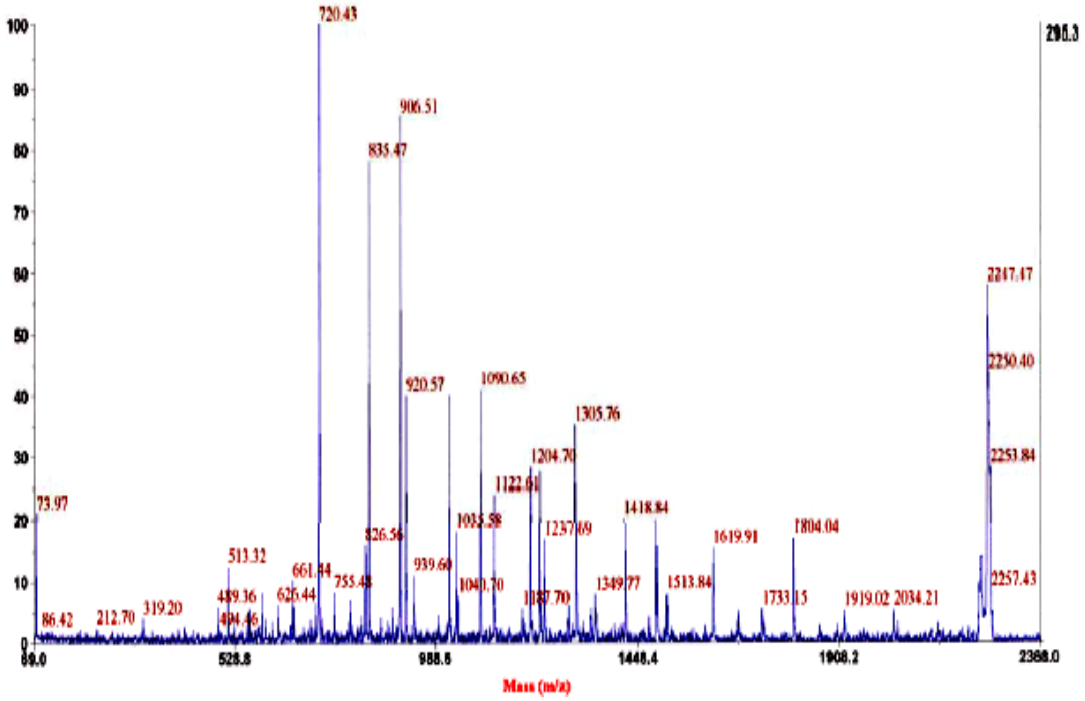

Figure 13: MALDI-MS/MS spectra of Sp D2 2244 Da peptide

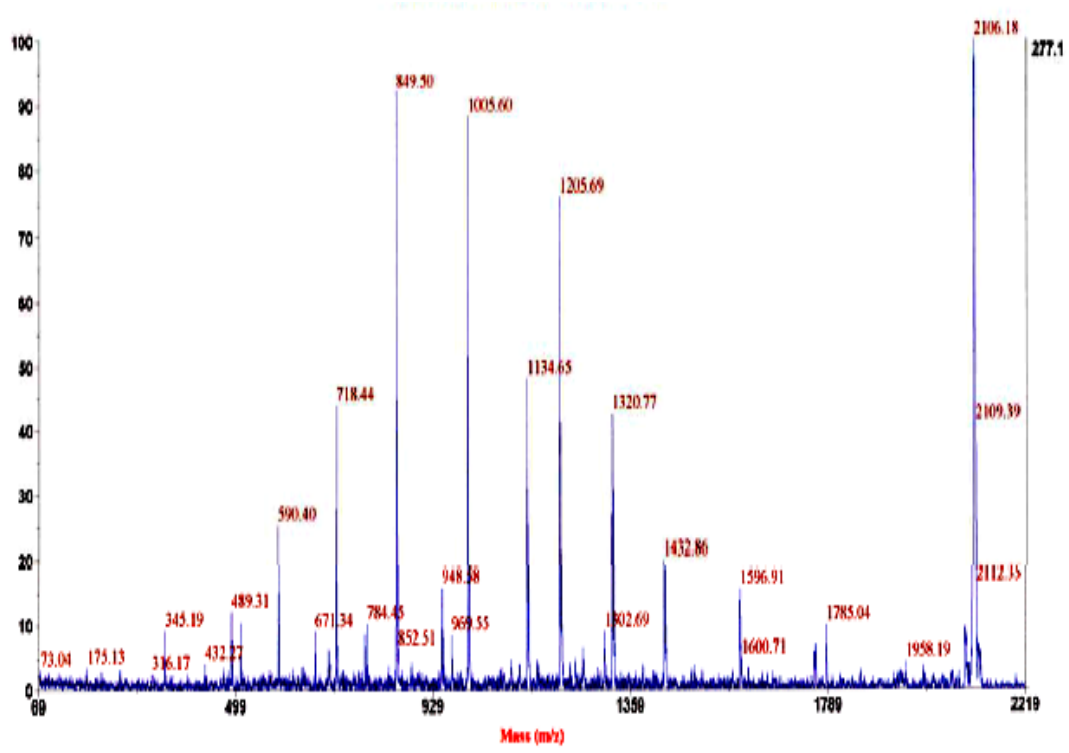

Figure 14: MALDI-MS/MS spectra of Sp D2 2102 Da peptide

Database Searches for Sequence Similarity With Sp D2 MS Results

The In-gel digests obtained from the band of interest were analyzed by MALDI-MS yielding a peptide mass fingerprint. The monoisotopic peptide masses obtained were searched against the SWISS-PROT, NCBInr and MSDB databases using the MASCOT search program. The resulting protein hits are scored using a probability based Mowse score. The score is -10*Log (P), where P is the probability that the observed match is a random event.

The results from the database searches sowed only one protein CagA (from *Helicobacter pylori)* received a marginal significant score from the NCBInr and MSDB databases but not from the Swissprot database. As a result, this is not considered a true protein match. No protein was identified as having a significant score when searched against *S. pneumoniae* data available in all three databases. The proteins listed as the top scoring proteins from all databases do not share any apparent similarities. The MALDI-MS and MS/MS results therefore did not provide a conclusive identification of the Sp D2 unknown protein.

N-terminal Sequence Data

Sp D2 protein samples were subjected to SDS-PAGE/blotting onto PVDF membrane and stained with Amido Black yielding a profile. The major band of interest was excised and sequenced using 18 Edman degradation cycles yielding an N-terminal sequence. WU-BLAST was used to search the Swall database with the N-terminal sequence data. There were no significant matches obtained from the database which included *S. pneumoniae* proteome. The only similarity shared by the proteins is that they

appear to be membrane bound. The N-terminal sequence obtained is as follows: DSSFDKLVADALGITAPA (18 amino acids)

Internal Sequence Data

A Trypsin In-gel digest was performed on the band of interest. The digest was fractionated by HPLC and purified peptides were subjected to 23 cycles of Edman degradation in order to determine internal sequences. The resulting sequence were analyzed in the same manner as the N-terminal sequence. Like the N-terminal sequence data, there were no significant matches and none from *S. pneumoniae* proteome. The internal sequences obtained are as follows:

VLNDAIEALTNAIANGTGSVDGK (23 amino acids), DKIDADFAGDLS (12 amino acids) and KAYSDAVYAVR (11 amino acids).

Serotype Studies and Additional Biochemical Characterization of *S. pneumoniae* Clinical Isolates

Serotyping is the golden standard of discriminating between *S. pneumoniae* strains based on their capsular type and is the basis of the current capsular polysaccharide vaccines. Serotyping of the clinical isolates was conducted using antisera to polysaccharide capsules representing all documented capsular types. Results showed that isolates in my study represented a wide variety of serotypes (Table 6). Three strains, Sp D-2, Sp Original and Sp G-5481 were non-typeable, which means that they could not be identified as having any of the documented capsular types. The most common serotype expressed is 14, exhibited by six strains.

The Optochin test and bile solubility tests are routinely used as biochemical tests for presumptive identification of *S. pneumoniae*. All strains except Sp G-5481 tested positive by the bile solubility and optochin test, this strain is also non-typeable. Sp Original showed variable results for the optochin test (Table 14).

TABLE 6: Results for Serotype Studies, Bile Solubility and Optochin Test

[a]Strain	Gram stain	Serotype	Bile solubility	Optochin test
Sp D-1	gram +	9V	+	+
Sp D-2	gram +	[b]non-typeable	+	+
Sp D-3	gram +	6A	+	+
Sp D-4	gram +	19F	+	+
Sp D-5	gram +	14	+	+
Sp D-6	gram +	6B	+	+
Sp D-7	gram +	19F	+	+
Sp D-8	gram +	14	+	+
Sp D-9	gram +	14	+	+
Sp D-10	gram +	14	+	+
Sp D-11	gram +	14	+	+
Sp D-12	gram +	14	+	+
Sp G-25053	gram +	9V	+	+
Sp G31159	gram +	18C	+	+
Sp G-5481	gram +	[b]non-typeable	-	-
Sp G-3143a	gram +	23F	+	+
Sp Original	gram +	[b]non-typeable	+	+/-

[a]Abbreviation for source strains Sp D = Duke strains, Sp G = Grady strains
[b]Non-typeable = strains that cannot be serotyped and fit the standard definition of non-typeable *S. pneumoniae* based on the other test results in the above table

Additional Antibiotic Susceptibility Testing of Sp D2

TABLE 7: Minimal Inhibitory Concentration (MIC) Results of Sp D2

Antibiotic	MIC (μg/ml)	Interpretation	Antibiotic Classification
Penicillin	4	R	β-lactam
Chloramphenicol	4	S	Chloramphenicol
Tetracycline*	0.25	S	Tetracycline
Erythromycin*	>2	R	Macrolide
Clindamycin*	0.06	S	Macrolide
Trimethoprim/Sulfamethoxazole*	8/152	R	Sulfonamide/antimetabolite
Cefotaxime (nonmeningitis)*	8	R	Cephalosporin/β-lactam
Cefotaxime (meningitis)	8	R	Cephalosporin/β-lactam
Ceftriaxone (nonmeningitis)*	4	R	Cephalosporin/β-lactam
Ceftriaxone (meningitis)	4	R	Cephalosporin/β-lactam
Cefepime (nonmeningitis)*	2	I	Cephalosporin/β-lactam
Cefuroxime	>4	R	Cephalosporin/β-lactam
Meropenem	1	R	Carbapenem/β-lactam
Imipenem*	0.5	I	Carbapenem/β-lactam
Levofloxacin*	1	S	Fuloroquinolone
Vancomycin	0.5	S	Glycopeptide

*Not appropriate for treatment of meningitis

Additional antibiotic susceptibility tests were performed on Sp D2, using antibiotics not studied in my initial thesis results. Sp D2 was found to be resistant to a majority of the antibiotics (Table 7). It exhibited resistance or intermediate resistance to

all cephalosporins and carbapenems tested. As mentioned earlier, cephalosporins and carbapenems are β-lactams like penicillin in that they all inhibit cell wall synthesis. Sp D2 is susceptible to four antibiotics, chloramphenical, tetracycline, clindamycin (a macrolide), levofloxacin and vancomycin. However, tetracycline, clindamycin and levofloxacin are not suitable for treatment of meningitis.

PCR of Pneumococcal Surface Antigen, Autolysin and Pneumolysin Genes

In order to further characterize Sp D2 and positively identify it as a *S. pneumoniae, s*everal PCR procedures were used to determine the presence of common *S. pneumoniae* virulence factors. The genes targeted were the pneumococcal surface protein *(psaA*) gene, autolysin (*lytA*), and pneumolysin (*ply*) genes (Table 8). These PCR studies were also performed on the other clinical isolates.

All strains were positive for the presence of pneumococcal surface antigen (*psaA*) gene. All strains also showed the presence of autolysin *(LytA*) gene. Two strains, Sp-D6 and Sp D-11 did not show an amplification of the pneumolysin (Ply) gene using outer primers but were positive with the inner primers. Two of the Grady strains Sp G-31159 and G-5481 did not appear to have the Pneumolysin gene based on the use of two sets of primers.

Arbitrarily primed PCR was also run on Sp-D2 and results indicated that it is a *S. pneumoniae*.

TABLE 8: PCR results for *PsaA, LytA,* and *Ply*

[a]Strain	Pneumococcal surface protein (*PsaA)*	Autolysin *(LytA)*	Pneumolysin [b]outer *(Ply)*	Pneumolysin [c]inner *(Ply)*
Sp D-1	+	+	+	+
Sp D-2	+	+	+	+
Sp D-3	+	+	+	+
Sp D-4	+	+	+	+
Sp D-5	+	+	+	+
Sp D-6	+	+	-	+
Sp D-7	+	+	+	+
Sp D-8	+	+	+	+
Sp D-9	+	+	+	+
Sp D-10	+	+	+	+
Sp D-11	+	+	-	+
Sp D-12	+	+	+	+
Sp G-25053	+	+	+	+
Sp G31159	+	+	-	-
Sp G-5481	+	+	-	-
Sp G-3143a	+	+	+	+
Sp Original	+	+	+	+

[a]Abbreviation for source strains Sp D = Duke strains, Sp G = Grady strains
[b]outer = outer primers used in the nested PCR
[c]inner = inner primers used in the nested PCR

Correlation Studies Between Serotypes and PCR of *S. pneumoniae* Virulence Genes

There was no particular correlation between any specific serotype and the PCR results (Table 9). The pneumococcal surface protein (*PsaA)* gene appears to be present in all serotypes. This correlates with observation from other studies discussed earlier. All serotypes also showed the presence of autolysin (*lytA)* gene. The pneumolysin gene (*ply*) could not be amplified using nested PCR outer primers in two isolates with serotype 6B (Sp D-6) and serotype 14 (Sp D-11), but could be amplified using inner primers. Two isolates Sp G-31159 (serotype 18 C) and Sp G-5481 (non-typeable) did not appear to have the pneumolysin gene.

Correlation Studies Between Serotypes and Occurrence in Drug Resistance, Pediatric and Adult Infections.

Table 10 represents correlation studies between serotypes and their common occurrence associated with drug resistance, pediatric infections and adult infections. Other studies have shown that the most important pediatric serotypes are 6A, 14, 19F and 23Fs. In adults however, serotypes 3, 19F and 6A accounted for only 31% of the isolates (2). The serotypes 6B, 9V, 14, 19A, 19F, and 23F cause most drug-resistant infections in the United States (36).

My results indicate that most of the clinical isolates have serotypes associated with drug resistance, and three of the ones not grouped are non-typeable. These results correlate with the susceptibility profiles presented above, majority of the clinical isolates were resistant to at least one antibiotic. Majority of the isolates also have serotypes frequently associates with pediatric infections which is expected since they were isolates

from Otitis Media cases. Only three isolates (Sp D3, Sp D4, and Sp D7) have serotypes associated with adult infections.

TABLE 9: Correlation Between Serotypes and PCR of Virulence Genes

[a]Strain	Serotype	Pneumococcal surface protein (*PsaA)*	Autolysin *(LytA)*	Pneumolysin [b]outer *(Ply)*	Pneumolysin [c]inner *(Ply)*
Sp D-1	9V	+	+	+	+
Sp D-2	[b]non-typeable	+	+	+	+
Sp D-3	6A	+	+	+	+
Sp D-4	19F	+	+	+	+
Sp D-5	14	+	+	+	+
Sp D-6	6B	+	+	-	+
Sp D-7	19F	+	+	+	+
Sp D-8	14	+	+	+	+
Sp D-9	14	+	+	+	+
Sp D-10	14	+	+	+	+
Sp D-11	14	+	+	-	+
Sp D-12	14	+	+	+	+
G-25053	9V	+	+	+	+
G-31159	18C	+	+	-	-
G-5481	[b]non-typeable	+	+	-	-
G-3143a	23F	+	+	+	+
Original	[b]non-typeable	+	+	+	+

[a]Abbreviation for source strains Sp D = Duke strains, Sp G = Grady strains
[b]outer = outer primers used in the nested PCR
[c]inner = inner primers used in the nested PCR

TABLE 10: Correlation Between Serotypes and Occurrence in Drug Resistance, Pediatric and Adult Infections.

[a]Strain	Serotype	Serotype associated with drug-resistance	Serotype associated with pediatric infections	Serotype associated with adult infections
Sp D-1	9V	+		
Sp D-2	[b]non-typeable			
Sp D-3	6A		+	+
Sp D-4	19F	+	+	+
Sp D-5	14	+	+	
Sp D-6	6B	+		
Sp D-7	19F	+	+	+
Sp D-8	14	+	+	
Sp D-9	14	+	+	
Sp D-10	14	+	+	
Sp D-11	14	+	+	
Sp D-12	14	+	+	
Sp G-25053	9V	+		
Sp G31159	18C			
Sp G-5481	[b]non-typeable			
Sp G-3143a	23F	+	+	
Sp Original	[b]non-typeable			

[a]Abbreviation for source strains Sp D = Duke strains, Sp G = Grady strains
[b]Non-typeable = strains that cannot be serotyped and fit the standard definition of non-typeable *S. pneumoniae* based on the other test results in the above table

Assay for Degradation of β-Lactams by Sp D-2

Since Sp-D2 exhibited the highest level of resistance to β-Lactam antibiotics and due to the presence of the unique protein, we used an assay to determine if Sp-D2 was possibly degrading β-Lactams. Production of β-Lactamases is the common mechanism of breaking down β-Lactams by certain organisms such as *Moraxellae catarrhalis*. In order to treat infections caused by β-Lactamase producing strains, antibiotics combined with β-Lactamase inhibitors such as clavulanate acid are administered. The most common treatment being amoxicillin-clavulanate acid, otherwise known as augmentin. For the assay we chose amoxicillin without the inhibitor. Results of our study showed that when Sp-D2 is grown in the presence of amoxicillin levels below the MIC, the organism grows. This growth media was filtered to remove the Sp D2 cells and further diluted to be used to culture Sp Original. The amoxicillin concentration remained the same and Sp Original growth was inhibited. When the same assay was repeated with the presence of a *Moraxellae catarrhalis* strain that has been shown to produce β-Lactamase, Sp Original grew in all tubes. This indicates that Sp-D2 did not break down amoxicillin while the positive control *M. catarrhalis* did.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Antibiotic Susceptibilities and SDS-PAGE Analysis

The first phase of my research focused on *S. pneumoniae* and its increased level of resistance to β-lactam antibiotics. This increase in resistance has been attributed mostly to horizontal gene transfer since this species is a naturally transformable organism.

All strains in this study were characterized using SDS-PAGE. This is an important molecular epidemiological tool because it generates a profile that can distinguish between strains that otherwise may have very similar characteristics based on phenotypic and biochemical tests (75). The SDS-PAGE profiles yielded some major bands of proteins present in all strains. However, unique bands were found in certain strains that may be used to distinguish one strain from the others. Sp D2 especially exhibited a unique highly concentrated band with a molecular weight of approximately 50 kDa.

Another aspect of the research was to determine the susceptibility patterns of these organisms from different sources and geographical regions. Bacterial pathogens have become increasingly resistant to commonly used antibiotics. This increased resistance to antimicrobial agents is occurring in many countries and has complicated medical and economic health care decision-making. Some of these microorganisms are not easily treated with narrow spectrum antibiotics resulting in the use of broader and

usually more expensive agents (46). It has thus become necessary to stay up-to-date on bacterial antibiotic resistance patterns because these change over time. It is therefore recommended that there should be routine susceptibility testing of organisms and their mechanisms of resistance studied (46). This allows the best balance in avoiding unnecessary use of broad-spectrum antibiotic therapy while aggressively treating resistant pathogens (46).

The β-lactams are by far the most widely used and efficacious of all antibiotics. The current choice of treatment for pneumococcal infections are β-lactams, most of which are penicillin derivatives. The evolution of frequent, high-level penicillin resistance in the pneumococcus has led to a reappraisal of the treatment of common respiratory tract infections. This includes a return to routine susceptibility testing of pneumococcal clinical isolates, looking for penicillin resistance in pneumococci and re-examination of the utility of pneumococcal vaccine in prevention of clinical infections (46).

Antibiotic susceptibility test results of this study correlate with this phenomena of increased high level resistance by *S. pneumoniae*. There were eleven clinical isolates used in this study plus the six strains used in the formulations of one widely used pneumococcal vaccine. Ten out of the eleven clinical isolates showed resistance to Ampicillin, with five of them being extremely high, such that there were no zones of inhibition in the Kirby Bauer test. Only one isolate (Sp D2) showed resistance to all the β-lactam antibiotics used. All strains were however still susceptible to Vancomycin, the only none β-lactam antibiotic used in my study.

All strains were negative for the production of a β-lactamase enzyme using the Cefinase disk test. These results correlate with the known observation that to this date, no β-lactamase producing *S. pneumoniae* strain has been described (9). On the other hand, over 90% of *Moraxella catarrhalis* strains worldwide are β-lactamase positive (9). This is an interesting observation since both organisms co-exist in the upper respiratory tract. With *S. pneumoniae* being a natural transformant, it is feasible that they would pick up some β-lactamase encoding genes from these organisms existing in such close proximity. This has however not been observed. It has been suggested that *Moraxella catarrhalis* may provide indirect protection to *S. pneumoniae* via its β-lactamase production especially in a biofilm setting (9). So far, the most common method of *S. pneumoniae* resistance to β-lactam antibiotics is by altered penicillin-binding proteins.

β-lactam Resistance Mechanisms Study

Another aspect of my research included experiments that dealt with β-lactam resistance mechanisms. In an attempt to explain the mechanism of resistance to β-lactam antibiotics of the strains used in this study, BOCILLIN FL was used. BOCILLIN FL is a commercially available fluorescent penicillin (95). The concept of this study is based on the fact that most *S. pneumoniae* strains that are resistant to β-lactams have altered penicillin-binding proteins (PBPs) that would have less binding affinity to the antibiotics (27). Since BOCILLIN FL is a fluorescent penicillin, it would bind to the penicillin-binding proteins which can be separated by SDS-PAGE and visualized under UV light. The altered PBPs would have a less affinity for BOCILLIN, hence less fluorescent intensity or none at all.

Preliminary results on the use of BOCILLIN FL to detect PBPs utilized two strains (Sp 95 and Sp G25053) based on their susceptibility results. These susceptible strains were chosen in order to use PBPs that do not have less binding affinity to BOCILLIN FL to optimize the system. There were two distinct bands visible in both strains. There were other bands scattered in the gel also. This shows that BOCILLIN FL was able to bind penicillin-binding proteins in the membrane fractions of Sp 95 and Sp G25053. These results however were not conclusive since there was no control present. In order for further studies to be conducted using BOCILLIN FL, a control would need to be obtained, such as purified PBPs.

Another approach used in this study on *S. pneumoniae* β-lactam resistance was the generation of step mutants. The mutants were generated in an attempt to study the mechanisms of resistance that the mutants may have developed. *S. pneumoniae* strains were exposed to increased levels of ampicillin not unlike what may occur in a natural environment such as hospitals or livestock farms. Previous studies have used penicillin to generate step mutants in the same manner. Severin et al (73), generated mutants with 100 fold increase to penicillin (from 0.02 to 2.0 μg/ml) and 20 fold increase (from 0.5 to 10 μg/ml). The highest resistant *S. pneumoniae* strains isolated have generally had MICs to penicillin of 8 – 16 μg/ml (72). In this study, Sp G31159 was stepped up to 7 μg/ml from 0.39 μg/ml and Sp Original was stepped to 5 μg/ml from 0.195 μg/ml ampicillin. The resistance break point for Ampicillin is 4 μg/ml. These mutants were preserved for future studies.

Natural Transformation of *S. pneumoniae*

Another aspect of my research was to relate the antibiotic resistance of *S. pneumoniae* to horizontal gene transfer. One important process used by *S. pneumoniae* to acquire resistance genes is by natural transformation.

Competence for transformation in S*treptococci* is not constitutive, as it is in *Neiserria* species, but is regulated by a quorum-sensing system encoded by two genetic loci, comCDE and comAB (45, 89). To date competence in *S. pneumoniae* has been demonstrated at densities ranging from ($10^6 – 10^8$) (23, 64). It has been suggested that in the natural environment (the nasopharynx) this could be important because if the cell density critical for competence is close to that which triggers host defenses, competence could then serve for adaptation of *S. pneumoniae* to host defense-generated stress (15).

Other factors have been described that affect the expression of competence by *S. pneumoniae*. It has been shown that an increase of the pH of the medium continuously moved the competence peak to earlier exponential phase and thus to lower cell densities (48). A slightly basic pH seems to be optimal (7.4 to 8.0) and no competence has been detected at pH less than 7.0 (23, 48, 49, 81, 83). The reason being that the (Competence Stimulating Peptide) CSP is more active at alkaline than neutral pH (48). It has also been shown that in *S. pneumoniae*, Ca^{2+} is essential for growth, competence development, and autolysis (48, 49). Competence is induced optimally at 1 mM Ca^{2+}.

Another major factor affecting *S. pneumoniae* competence is serum. It was established by Hotchkiss and Tomasz in their pioneering work that serum albumin was required for *S. pneumoniae* natural transformation (34, 81). To date, the addition of serum has been maintained in transformation experiments (4, 29, 57-59). Finally, the

presence of capsule has been shown to abolish competence for natural genetic transformation in *S. pneumoniae* (8, 48, 66).

Sp D2 Unknown Protein Analysis

Initial antibiotic susceptibility tests revealed that the clinical isolate Sp D2 from Duke University is resistant to all the β-lactam antibiotics that were tested namely; ampicillin, amoxicillin, ceftazidime, and penicillin G. The whole cell SDS-PAGE protein profiles also revealed a protein band approximately 50 kDa that was unique to Sp D2.

A literature study was first conducted on PBPs in an attempt to determine if the unique protein of Sp D2 was in the size range of the known *S. pneumoniae* PBPs (Table 1). All the *S. pneumoniae* PBPs that have been shown to contribute to β-lactam resistance have sizes between 70 kDa and 98 kDa. Only one, PBP3 is 43 kDa and has been shown not to participate in resistance development. The protein of interest being approximately 50 kDa, did not fall within the resistance determining PBPs. A similar study was performed on the known virulence factors of *S. pneumoniae* and this protein still did not fall within any known virulence protein size ranges.

SDS-PAGE of Cytoplasmic Membrane Fractions

Since PBPs are membrane bound enzymes, the membrane proteins of several of the strains were isolated including Sp D2 in attempt to determine if the unique protein is membrane bound. The membrane fractions were run on an SDS-PAGE gel (see Figure 6). Initial studies (see Figure 3, Lane 3) revealed a highly concentrated band of approximately the same size as the protein of interest present in the Sp D2 lane but not in

any of the other strains tested. These preliminary studies indicated that the unique Sp D2 protein was membrane bound even though it did not appear to be within the size range of the PBPs. The next steps were to further characterize the unique protein.

MALDI-MS and MS/MS Peptide Analysis and Edman Degradation Sp D2 Protein

The protein of interest was excised from the membrane fraction SDS-PAGE gels and processed using an In-Gel trypsin digest kit. This kit allows the extraction of the protein band with subsequent trypsin digest. Trypsin cleaves after Lysine and Arginine residues (except if they are present before Proline) therefore producing specific fingerprints for specific proteins. The resulting peptides were then analyzed by MALDI-MS (matrix-assisted laser desorption and ionization mass spectrometry). This is a mass spectrometry method that can, with great precision, determine the molecular weights of biological specimens. The peptide digests are mixed with the matrix material and then ionized with a laser. The longer it takes for the ionized material to travel to the detector, the larger its molecular weight. A spectra is thus produced (see Figures 9-14) reflecting the peptide masses and their relative abundance. The initial MS spectra is of the Sp D2 intact peptides. From this spectra, the five most abundant peaks were selected (an automated procedure) and further fragmented by MS/MS (tandem ms). Both types of spectra display fingerprints unique to specific proteins and peptide sequences.

Three database were used to identify proteins by sequence similarity searches using the peptide sequences produced by the interpretation of the tandem mass spectra. The resulting protein hits are scored using a probability based Mowse score.

Mass spectrometry analysis indicated that only one protein CagA (from *Helicobacter pylori)* received a marginal significant score from the NCBInr and MSDB databases but not from the Swissprot database. As a result, this is not considered a true protein match. No protein was identified as having a significant score when searched against *S. pneumoniae* data available in all three databases. The proteins listed as the top scoring proteins from all databases do not share any apparent similarities. The MALDI-MS and MS/MS results therefore did not provide a conclusive identification of the Sp D2 unknown protein. Attempts to further analyze the MALDI-MS and MS/MS data and construct sequence tags by Dr Gorin and Dr, Day of Oakridge labs have been unsuccessful.

N- terminal and internal sequence data generated by Emory University microchemical facilities did not receive any significant hits with any database. We can therefore conclude that the Sp-D2 is a unique protein that has not been described before and is not present in the databases.

Phase II of Research Studies

Due to the inconclusive results obtained from the sequencing of the Sp D2 protein, my research was refocused hence the second phase. It was determined that cloning and sequencing of the Sp D2 unknown gene was considered too time consuming and expensive.

We therefore initiated a collaboration with Dr. Facklam and the Active Bacterial Core Surveillance/Emerging Infections Program Network at CDC to further study Sp D2 and continued the studies to the other clinical strains presented in my thesis as well. We

further characterized Sp D2 to conclusively establish its taxonomic classification by, serotyping and analysis by PCR for the presence of pneumococcal surface protein *(PsaA*) gene, autolysin (*lytA*), and pneumolysin (*ply*) genes.

S. pneumoniae is a member of the oral normal flora, among other *Streptococcus* species (*S. oralis, S. mitis*) which exhibit alpha hemolysis (91).Three phenotypic tests (optochin susceptibility, bile solubility, and agglutination with the antipneumococcal polysaccharide capsule antibodies) were previously used to differentiate classical *S. pneumoniae* from those species. Thus, atypical or variant *S. pneumoniae* strains may be improperly classified on the basis of phenotypic characteristics.

Additional Antibiotic Susceptibility Testing of Sp D2

Additional antibiotic susceptibility tests were performed on Sp D2, using antibiotics not included in my initial thesis results. Sp D2 was found to be resistant to erythromycin, trimethoprim/sulfamethoxazole, and all β-lactams (see Table 6). Strains that are non-susceptible to three or more drug classes are considered to be multiply resistant (92).

Sp D2 was found to be susceptible to four antibiotics, chloramphenical, tetracycline, clindamycin (a macrolide), levofloxacin and vancomycin. However, tetracycline, clindamycin and levofloxacin are not suitable for treatment of meningitis. This leaves only chloramphenical and vancomycin. Unfortunately, vancomycin must be administered intravenously to treat pneumococcal infections and its use tends to select for vancomycin-resistant Enterococci. The fluoroquinolones have drawbacks due to the ease of acquiring resistance by changing only one nucleotide in the DNA gyrase and relatively expensive (46). Thus an infection or outbreak caused by Sp D2 would present a serious

treatment problem. This being one of the reasons of interest by individuals of the Pneumococcal Molecular Epidemiology Network (PMEN).

Serotype Studies and Additional Biochemical Characterization of *S. pneumoniae* Clinical Isolates

In order to further characterize Sp D2 and positively identify it as a *S. pneumoniae*, several characterization studies were undertaken. Additional biochemical tests used for the presumptive identification of *S. pneumoniae* were performed as well as serotyping studies on Sp D2 and all other clinical isolates in my research.

Additional biochemical test results showed that was Sp D2 was positive by the optochin and bile solubility tests. These results are typical for *S. pneumoniae* strains. All the other strains except Sp G-5481 also tested positive by the bile solubility and optochin test. Sp Original showed variable results for the optochin test.

Sp D2 was shown to be non-typeable. The most common serotype expressed by our clinical isolates was serotype 14 (six strains). The distribution of types isolated from adults differs substantially from that of types isolated from children. The most important pediatric serotypes (6A, 14, 19F and 23F) were responsible for almost 60% of all infections (2). In adults however, serotypes 3, 19F and 6A accounted for only 31% of the isolates (2). The serotypes 6B, 9V, 14, 19A, 19F, and 23F cause most drug-resistant infections in the United States (36).

Two of our strains were serotype 19F; two were serotype 9V, and one was serotype 23F. It is significant to note that certain serotypes (6B, 9V, 14, 19A, 19F, and 23F) have dominated the multidrug-resistant *S. pneumoniae* clones that have appeared

with different capsular serotypes. Such variants are thought to arise through natural transformation involving recombinational replacements with and around the capsular biosynthesis (*cps*) locus (17). Genes for the two PBPs are located on either side of the *cps* genes. Trzcinski et al., suggests that in natural habitats within the body, selection by host immunity and selection by antibiotics may be interrelated because of he effect of linkage of resistance determinants and capsule locus (85). However, it is significant to note that our strain (Sp D2) is nonencapsulated and multidrug resistant.

Little information is available in the literature on the epidemiology of nonencapsulated *S. pneumoniae* populations. Whatmore et al., described a group of nonencapsulated strains that appeared genetically closely related to typical encapsulated strains (91).

PCR Studies of Pneumococcal Surface Antigen, Autolysin and Pneumolysin Genes

Although pneumococcus is the most common bacterial cause of pneumonia in children and adults worldwide, the diagnosis of pneumococcal pneumonia is difficult to establish. Blood cultures are positive in only 20%-30% of adults and less than 10% in children with pneumococcal pneumonia (10, 70). Sputum is also difficult to obtain and young children do not produce sputum. Further more, sampling via bronchioscope is laborious. As a result, rapid and reliable methods for diagnosis of pneumococcal pneumonia have been developed using PCR (polymerase chain reaction). The genes that have been targeted are the genes encoding pneumolysin, autolysin, and pneumococcal surface protein (69).

As a result of inconclusive identification of the unique Sp D2 protein by MALDI-MS and Edman degradation studies, it was necessary to further characterize the strain and positively identify it as a *S. pneumoniae*. PCR studies were thus undertaken targeting common *S. pneumoniae* virulence encoding genes. The genes targeted were the pneumococcal surface protein *(psaA*) gene, autolysin (*lytA*), and pneumolysin (*ply*) genes.

All strains were positive for the presence of pneumococcal surface antigen (p*saA*) gene. My *psaA* results correlate with the study of Morrison et al that demonstrated the presence of *psaA* in all 90 serotypes tested, and that there was lack of amplification of heterologous organisms. This suggested that PCR amplification of *psaA* could be a very useful tool for detection of pneumococci and diagnosis of disease. This also shows that a protein common to all serotypes with genetic and immunologic similarity has implications for both vaccine studies and diagnostics development. It means that the use of an immunogenic common protein as a vaccine could eliminate the need for multiple capsular types in a pneumococcal vaccine and additionally elicit a memory response, which occurs only with protein-based vaccines (61).

All strains also showed the presence of autolysin *(LytA*) gene. Two strains, Sp-D6 and Sp D-11 did not show an amplification of the pneumolysin (Ply) gene using outer primers but were positive with the inner primers. Two of the Grady strains Sp G-31159 and G-5481 did not appear to have the pneumolysin gene based on the use of two sets of primers. There was no particular correlation between specific serotypes and gene amplified in this study. Although Sp G-5481 was non-typeable and did not appear to have the pneumolysin gene, it was determine to still fit the criteria of a *S. pneumoniae*

In clinical laboratories, identification of *S. pneumoniae* from other streptococci is important especially in cases where strains are not able to be serotyped. If a strain cannot be serotyped, an arbitrarily primed PCR with a single primer M13 universal is used as a method to distinguish *S. pneumoniae* from other upper respiratory tract streptococci (54). Sp D2 was positive by the arbitrarily primed PCR method. Although Sp D2 is non-typeable, the additional biochemical tests as well as all the PCR studies positively identify Sp D2 as a *S. pneumoniae*.

My thesis research has resulted in several significant findings: (i.) We have characterized Sp D2 as a nonencapsulated clinical isolate, that exhibits high level of resistance to erythromycin, trimethoprim/sulfamethoxazole, and several β-lactam antibiotics. These findings are sonsistent with the established criteria for a multidrug resistant strain. Strains that are non-susceptible to three or more drug classes are considered to be multiply resistant (92). (ii.) We have also partially characterized a membrane-bound protein in Sp D2 that is not associated with any PBPs., and (iii.) We have confirmed Sp D2 as expressing *PsaA* that encodes for the pneumococcal surface antigen A, the *LytA* that encodes autolysin, and the *Ply* that encodes pneumolysin; all of which establish criteria for classifying clinical isolates in the species of *S. pneumoniae*. Thus, these data suggest that Sp D2 may be an emerging clone that has acquired genes for the synthesis of a unique protein.

Sumary of Major Conclusions

1. Sp D2 can be classified as a multi-drug resistant strain.
2. Sp D2 is a non-typeable (does not react with any anticapsular antibody). Thus current polysaccharide vaccines would not be effective against Sp D2 infections.
3. Sp D2 showed the presence of pneumococcal surface antigen A, autolysin and pneumolysin encoding genes.
4. Sp D2 was positive for bile solubility and optochin.
5. Thus Sp D2 can be conclusively identified as a *S. pneumoniae.*

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